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BIOLOGICAL BULLETIN

SEXES IN THE CYNIPIDÆ AND MALE-PRODUCING AND FEMALE-PRODUCING LINES.[1]

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1. Introduction.

In certain groups of insects there occurs an alternation of generations in which a bisexual generation alternates with one or more parthenogenetic or agamic generations. Thus in Aphids and Phylloxerans such an alternation occurs, and in many of the gall wasps, or Cynipidæ, a similar condition is found. In the case of Phylloxerans, Morgan has pointed out that in some species (*e.g.*, *P. fallax*) a single agamic female lays (probably) both male-producing and female-producing eggs, while in other species (*P. caryæcaulis*) a given agamic female lays but one kind of egg. Her progeny therefore will be either all females or else all males. I have found similar conditions to exist among the Cynipidæ, and it is the purpose of this paper to give the essential facts.

The cytological basis of sex-determination in the Aphids and Phylloxerans is very well worked out, but the same can not be said for the Cynipidæ. However, the facts already known are sufficient to indicate that the males of all species are haploid, while all females are diploid. Males develop from unfertilized eggs in which the number of chromosomes has been reduced during maturation. Females develop either from fertilized eggs or from unfertilized eggs in which reduction has not taken place (Doncaster).

This means that the primitive species, which usually have a single bisexual generation a year, develop by facultative parthenogenesis. In such species all eggs are alike and all undergo the typical maturation divisions with reduction. If the egg is ferti-

[1] Contributions from the Zoölogical Laboratory, University of Texas, No. 210.

lized it produces a female (diploid), but if unfertilized it develops into a male (haploid). It is not known whether the unimpregnated female can lay both fertilized and unfertilized eggs, as is the case in the queen bee, or whether the unimpregnated female alone lays unfertilized eggs.

In species showing alternating generations the agamic females are the product of the fertilized eggs of the bisexual generation, but all of the individuals, both males and females, of this generation develop from unfertilized eggs laid by agamic females. As we shall show, in some species the female lays both male-producing and female-producing eggs, while in other species a given female lays but one kind of egg. Finally, in some of the more highly specialized Cynipidæ males are never found, and development is exclusively by parthenogenesis.

In breeding out various species of Cynipidæ for other purposes, a large number of data on sex ratios has incidentally been accumulated. From this we may select such cases as have a bearing on the questions outlined above. The plan has been followed of collecting single, isolated galls and breeding out all of the individuals from each gall. In this way it is possible largely to eliminate the danger of breeding from a gall which has been produced from the eggs of two or more females.

2. Sexes in the Primitive Genera.

Two genera of the primitive Cynipidæ have been investigated here—the genera *Aulacidea* and *Aylax*. Two species belonging to the first genus have been bred out. Both of these species infest the wild lettuce, *Lactuca ludoviciana*. These are *Aulacidea bicolor radialis* and *A. tumida conjuncta* (Kinsey, MS.).[2] *Bicolor* does not produce a gall but inhabits the pith of the stem. *Tumida* produces a distinct gall on the lettuce, usually in the form of a twisted swelling at the top of the stalk.

I have reared 20 females and 15 males of *A. bicolor*, and since it is one of the most primitive species, males and females are expected to appear in about equal numbers.

I have bred out 231 individuals from the galls of *A. tumida*.

[2] I am indebted to Dr. A. C. Kinsey for the identification or description of the species of Cynipidæ mentioned in this paper.

Of this number 51 only, or about 23 per cent., are males. In breeding from single isolated galls, it is found that each gall yields both sexes (Table I.).

TABLE I.

Aulacidea tumida conjuncta.

Galls.	Females.	Males.	Galls.	Females.	Males.
A.....	11	1	D.....	2	2
B.....	6	1	*E*.....	5	1
C.....	5	4	Mass	151	42
Totals.....	180	51	Per cent. of males, 22.51		

The genus *Aylax* is represented by a single species, *Aylax laciniata compacta* (Kinsey, MS.). It produces clusters of galls on the receptacles of the flowers of the rosinweed, *Silphium albiflorum*. The galls are formed in the sterile flowers toward the center of the disc. A total of 405 individuals of this variety have been bred out. About 45 per cent. of these are males, indicating that the sexes occur in about equal numbers.

TABLE II.

Aylax laciniata compacta.

Galls.	Females.	Males.	Galls.	Females.	Males.
A.....	2	3	*G*.....	2	0
B.....	2	0	*H*.....	4	1
C.....	4	0	*I*.....	7	0
D.....	3	0	*J*.....	2	2
E.....	5	3	*K*.....	1	1
F.....	2	4	Mass	192	165
Totals.....	226	179	Per cent. of males, 44.19		

The rearings from isolated galls are shown in Table II. Of the eleven galls bred from, six yielded individuals of both sexes and five those of one sex. On account of the numerous parasites, it is difficult to secure more than a few cynipids from each flower head. Thus 64 parasites and only 48 cynipids were bred out from the eleven galls.

The genus *Rhodites* is generally regarded as one of the primitive, in that none of its species is known to have an alternating genera-

tion. However, in some respects it gives evidence of high specialization, especially in its concentration on the genus *Rosa*. Its interest in this connection is found in the fact that in many species the males are much fewer in numbers than the females. In certain species the males do not exceed two per cent., and are, according to Adler, superfluous. Kinsey ('20) believes that on the whole most of the species reproduce agamically, "with the males still existent but not usually abundant enough to fertilize many, if any, of the females." I have not been able to find any species of *Rhodites* at Austin.

3. Sex Ratios in Species with Alternating Generations.

Among the most primitive species with alternating generations are those found in the genus *Neuroterus*, but there are several other genera exhibiting the same condition. The few cases given below are selected with the view of bringing out the main points concerning the female- and male-producing lines of the agamic females.

a. Andricus operator austrior form austrior Kinsey.

This variety has been described by Dr. A. C. Kinsey ('22) from material collected here at Austin. It is a bisexual generation, and what is in all probability the alternate generation is an acorn gall on *Quercus marylandica*. From the acorn galls I have bred out six females. The bisexual galls occur on *Q. schneckii*. The gall is a large, compact mass of wool, within which are found clusters of seed-like capsules containing the larval cells. It involves the young stems, young leaf clusters, and flower clusters.

A total of 1,235 individuals have been reared. About 46 per cent. of these are males. The results of the breeding experiments are shown in Table III. The point of chief interest is the fact that a large majority of the galls yielded individuals of both sexes. Thirty-four of the forty-five galls bred from gave cynipids of mixed sexes, eight yielded females only, and three gave males only. There is no doubt that the majority of agamic females are capable of laying both male-producing and female-producing eggs. However, some of them lay but one kind of egg (Table III., galls *i–s*).

TABLE III.

Andricus operdtor austrior form austrior.

Galls.	Females.	Males.	Galls.	Females.	Males.
A.....	2	4	*X*.....	2	1
B.....	25	4	*Y*.....	2	2
C.....	103	15	*Z*.....	8	3
D.....	69	33	*a*.....	18	2
E.....	16	8	*b*.....	2	1
F.....	4	2	*c*.....	21	1
G.....	21	6	*d*.....	15	1
H.....	11	2	*e*.....	17	5
I.....	4	2	*f*.....	5	2
J.....	71	2	*g*.....	10	1
K.....	26	8	*h*.....	2	1
L.....	5	4	*i*.....	8	0
M.....	34	11	*j*.....	3	0
N.....	35	1	*k*.....	5	0
O.....	2	3	*l*.....	15	0
P.....	8	1	*m*.....	2	0
Q.....	4	1	*n*.....	7	0
R.....	8	2	*o*.....	21	0
S.....	26	4	*p*.....	4	0
T.....	3	1	*q*.....	0	51
U.....	2	2	*r*.....	0	118
V.....	2	1	*s*.....	0	138
W.....	4	2	Mass	19	146
Totals.....	671	564	Per cent. of males, 45.66		

b. Andricus maxwelli Bassett.

This species forms polythalamous galls at the ends of twigs of *Quercus stellata*. It is an ideal gall from which to test out the kind of eggs laid by the cynipid. The galls are scattered and frequently a single gall will be found on one tree. Under such conditions it is highly improbable that a single isolated gall would be the product of the layings of more than one female. The number of individuals emerging from a single gall varies from one to as high as 27.

I reared 474 individuals of this species. About forty-two per cent. of these are males. The results of the breeding tests are tabulated in Table IV. Sixteen galls yielded males, eighteen yielded females, and seven gave individuals of both sexes. As compared with the preceding, *maxwelli* shows a distinct increase in the number of agamic females which lay one kind of egg.

TABLE IV.

Andricus maxwelli.

Galls.	Females.	Males.	Galls.	Females.	Males.
A.....	4	23	*V*.....	6	0
B.....	13	14	*W*.....	2	0
C.....	4	7	*X*.....	4	0
D.....	11	4	*Y*.....	3	0
E.....	3	1	*Z*.....	0	12
F.....	1	3	*a*.....	0	4
G.....	1	1	*b*.....	0	1
H.....	1	0	*c*.....	0	5
I.....	25	0	*d*.....	0	4
J.....	10	0	*e*.....	0	22
K.....	20	0	*f*.....	0	15
L.....	8	0	*g*.....	0	11
M.....	14	0	*h*.....	0	4
N.....	6	0	*i*.....	0	2
O.....	8	0	*j*.....	0	4
P.....	25	0	*k*.....	0	9
Q.....	8	0	*l*.....	0	5
R.....	8	0	*m*.....	0	2
S.....	7	0	*n*.....	0	3
T.....	4	0	*o*.....	0	2
U.....	13	0	Mass	68	39
Totals.....	277	197	Per cent. of males, 41.56		

c. Andricus maxwelli var.

A distinct variety of the preceding makes a somewhat similar gall on the twigs of *Q. breviloba*. This variety has not as yet been described. It is No. 47 of my collection of Cynipidæ. I have been able to breed out 138 wasps of this species. The results are shown in Table V. Of the fifteen individual galls bred from, all but two gave individuals of one sex. This variety, therefore,

TABLE V.

Andricus maxwelli (variety).

Galls.	Females	Males.	Galls.	Females.	Males.
A.....	4	5	*I*.....	0	8
B.....	2	1	*J*.....	0	5
C.....	8	0	*K*.....	0	13
D.....	11	0	*L*.....	0	8
E.....	3	0	M.....	0	4
F.....	2	0	*N*.....	0	2
G.....	3	0	*O*	0	2
H.....	0	7	*Mass*	7	43
Totals.....	40	98	Per cent. of males, 71.01		

shows a further tendency for the agamic female to lay but one kind of egg.

d. Eumayria floridana texana (Kinsey, MS.).

This species produces a polythalamous gall at the end of roots of *Quercus schneckii*. Table VI. gives the results of the rearing

TABLE VI.

Eumayria floridana texana.

Galls.	Females.	Males.	Galls.	Females.	Males.
A.....	124	1	*F*.....	0	69
B.....	8	88	*G*.....	0	53
C.....	267	0	*H*.....	0	6
D.....	0	138	Mass	7	225
E.....	0	149	Totals.....	406	729
Total reared, 1135			Per cent. of males, 64.22		

tests. Of the 1,135 individuals bred out, 729 or 64 per cent. are males. Two of the eight isolated galls give both sexes, one yielding 124 females and a single male, the other, 8 females and 88 males. Each of the other six galls gave individuals of one sex. This species must have an agamic generation, for it has been observed that the females begin to oviposit in the leaves of the same tree immediately after emerging.

e. Belenocnema treatæ kinseyi form parallela (Kinsey, MS.).

We may now consider four species in which the individuals emerging from one gall are always of the same sex. This means that the agamic female must be either a male-producer or a female-producer.

The polythalamous galls of the bisexual generation of *Belenocnema treatæ* variety *kinseyi* occur on the rootlets of the common live oak, *Q. virginiana*. The galls of the agamic generation (*B. treatæ kinseyi* form *kinseyi*) are monothalamous and are found on the under side of the leaves of the same oak. The galls of the bisexual generation invariably yield cynipids of the same sex (Table VII.). The sexes occur in about equal numbers. Of the 677 individuals reared, 329 are males. This gives about 49 per cent. of males.

TABLE VII.

Belenocnema treatæ kinseyi form parallela.

Galls.	Females.	Males.	Galls.	Females.	Males.
A	7	0	*K*	0	7
B	9	8	*L*	5	0
C	0	33	*M*	6	0
D	10	0	*N*	22	0
E	2	0	*O*	8	0
G	7	0	*P*	23	0
H	0	16	*Q*	9	0
I	8	0	Mass	241	253
J	0	12	Totals	348	329
Total reared, 677			Per cent. of males, 48.59		

f. Neuroterus irregularis albipleuræ Kinsey.

This variety of *Neuroterus* forms galls on the young leaves of *Q. breviloba*. It is a typical bisexual generation, but the alternate or agamic generation is not known. The individual galls always yield wasps of one sex (Table VIII.). The sexes probably occur in equal numbers, although but 40 per cent. of the 902 wasps bred out are males.

TABLE VIII.

Neuroterus irregularis albipleuræ.

Galls.	Females.	Males.	Galls.	Females.	Males.
A	0	20	*J*	18	0
B	0	19	*K*	7	0
C	0	9	*L*	0	3
D	5	0	*M*	0	12
E	0	9	*N*	0	4
F	10	0	*O*	4	0
G	41	0	*P*	2	0
H	0	8	Mass	454	243
I	0	34	Totals	541	361
Total reared, 902			Per cent. of males, 40.02		

g. Neuroterus vernus evanescens Kinsey.

This variety has galls in the form of swellings on the ament stem of *Quercus breviloba*. The results of the rearings are seen in Table IX. Perhaps not enough galls were bred out to show conclusively that individuals of one sex always emerge from a single gall. However, in addition to the five galls listed in the table, my field notes state that mixed sexes have never been seen emerging from one gall.

TABLE IX.

Neuroterus vernus evanescens.

Galls.	Females.	Males.	Galls.	Females.	Males.
A.....	1	0	*D*.....	0	4
B.....	0	5	*E*.....	3	0
C.....	0	4	Mass	44	46
Totals.....	48	59	Per cent. of males, 55.14		

h. Plagiotrochus candidus (Kinsey MS.).

This species forms galls on the leaf petiole of *Quercus schneckii*. As a rule, a single cynipid emerges from one gall, but whenever there are two or more, they are of the same sex (Table X.).

TABLE X.

Plagiotrochus candidus.

Galls.	Females.	Males.	Galls.	Females.	Males.
A.....	2	0	*J*.....	1	0
B.....	1	0	*K*.....	0	2
C.....	3	0	*L*.....	1	0
D.....	1	0	*M*.....	1	0
E.....	1	0	*N*.....	4	0
F.....	2	0	*O*.....	1	0
G.....	0	1	*P*.....	1	0
H.....	0	1	Mass	11	72
I.....	3	0	Totals	33	76
Total reared, 109			Per cent. of males, 69.72		

i. Neuroterus niger pattersoni form pattersoni (Kinsey).

In all of the forms considered above the galls are polythalamous, or else clusters. It is interesting to know that in a species producing scattered monothalamous galls it is possible to test out the agamic female as to the kind of egg she lays. The opportunity to do this is found in *Neuroterus niger pattersoni*. Both generations are known. The galls of the agamic generation are found as small swellings on the leaves of *Q. breviloba*, and probably *Q. stellata* also, while those of the bisexual generation occur on the leaves of *Q. stellata*. The agamic females emerge in March and immediately oviposit their eggs on the underside of the post oak leaves. It was noted that once a female began laying her

eggs in a given leaf, she never moved to another leaf, but continued until all of her eggs were laid. This suggested the possibility of breeding out the bisexual individuals from separate leaves. The results of these tests are shown in Table XI. Of the 26 leaves thus tested, 22 gave cynipids of one sex, and four gave mixed sex (leaves *W–Z*).

TABLE XI.

Neuroterus niger pattersoni form pattersoni.

Leaf.	Females.	Males.	Leaf.	Females.	Males.
A	55	0	*O*	52	0
B	31	0	*P*	0	9
C	0	19	*Q*	0	9
D	14	0	*R*	0	10
E	0	21	*S*	0	12
F	0	9	*T*	0	24
G	0	6	*U*	0	4
H	4	0	*V*	0	2
I	0	16	*W*	9	58
J	4	0	*X*	12	1
K	0	11	*Y*	8	53
L	0	19	*Z*	1	21
M	0	6	Mass	468	465
N	21	0	Totals	683	775
Total reared, 1,458			Per cent. of males, 53.15		

It is highly probable that the four exceptions are due to the fact that more than one agamic female laid in each leaf. That this is so, is indicated by breeding out ten galls recently. These galls were carefully selected from a region in which the galls were very scarce. Care was taken to secure a single isolated leaf from each of ten trees. Each of these ten leaves gave wasps of one sex (leaves *M–V*, Table XI.). From this it may be concluded that the agamic female of this species lays but one kind of egg.

4. DISCUSSION.

The species listed above are a few of a rather large number in which the breeding tests indicate that the agamic females are of two types. The evidence shows clearly that among heterogenous species the function of laying one kind of egg by the agamic female has evolved from a condition in which such a female laid both male- and female-producing eggs.

The chief point of interest is whether it is possible to find

cytological evidence that would be adequate to explain this condition in the Cynipidæ. Doncaster ('16) has attempted to solve the question of the nature of the difference between the female-producing and male-producing females of the agamic generation of *Neuroterus lenticularis*, one of the heterogenous species found in Europe. He attacked the problem both by breeding experiments and by cytological investigations. He concludes from the results of his breeding tests that the two types of agamic females could not be due to a dimorphism of sperms produced by one male. He suggests two other possibilities: (1) That the two types of female may be due to two kinds of eggs laid by different sexual females; (2) or, if each sexual female mates with only one male, they may be due to two kinds of males which produce different spermatozoa.

Doncaster found no evidence in the spermatogenesis which would support the last suggestion. In studying the maturation of the eggs laid by the sexual females, he did find differences indicating that there might be two classes of eggs. However, he did not regard these differences sufficient "to correlate them with the sex-phenomena with any confidence." The question of the nature of the difference between the two types of agamic females in the Cynipidæ is therefore unanswered, and its solution awaits further study.

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FACTORS INFLUENCING THE RATE OF METABOLISM OF *ÆSHNA UMBROSA* NYMPHS.

MARY HONORA SAYLE.

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INTRODUCTION.

The respiratory exchange of an animal is of special physiological interest, because it affords a means of securing quantitative evidence regarding the metabolic processes taking place inside the living animal. It may be measured by ascertaining either the oxygen consumption or the carbon dioxide output. At present, methods for the determination of the latter have been greatly improved and are especially favorable for work on lower forms, such as insects. The literature dealing with metabolic rate as influenced by feeding, starvation and change of temperature and light, includes a number of papers on the metabolism of nsects, but little or nothing in the way of direct quantitative measurements of metabolism in dragon fly nymphs.

HISTORICAL.

Spallanzani (22) and Plateau (18) determined the vital limit of tolerance to high temperature of many species of insects. Spallanzani (23), Treviranus (25), von Linden (27), Weinland (28), and Fink (9) studied the gaseous exchange of various insects. Newport (15) and Sosnowski (21) determined the carbon dioxide output of insects in various stages of development. In the classical work of Regnault and Reiset (19), the carbon dioxide

output of May-beetles was determined. Bütschli (6) and Vernon (26) studied the effect of temperature upon the gaseous exchange of *Blatta orientalis* fed on different substances. Slowtzoff (20), Batelli and Stern (2), Parhon (17), and Dirken (7) also observed the influence of temperature on the respiratory exchange of such insects as ants, bees, bettles, silkworms, and flies. Many experiments have been performed on the gaseous exchange of the silkworm. Of these the most outstanding ones are those of Luciani and Piutte (14), Luciani and Lo Monaco (13), and Farkas (8).

Loeb and Northrop (12), Bodine and Orr (5), and Northrop (16) studied the respiratory metabolism of *Drosophila* cultures. Bodine (3) (4) studied the water content and the rate of metabolism of active and hibernating grasshoppers. Studies on the influence of light on the metabolic rate were made by Loeb (11) on the chrysalids of certain butterflies and moths and by Allee and Stein (1) on the May-fly nymphs.

Perhaps the most useful compilation of data on metabolism is to be found in Krogh's (10) monograph on "The Respiratory Exchange of Animals and Man." This work includes the results of several original experiments on insect pupæ, particularly on chrysalids of *Tenebrio molitor*.

The purpose of the present study was to determine the effect of starvation, darkness, and temperature upon the carbon dioxide output of *Æshna umbrosa* nymphs. These nymphs are easily obtained and live successfully under general laboratory conditions. They offer especially desirable material for the study of the respiration in a truly aquatic form. Acknowledgment is made to Prof. A. S. Pearse for suggesting the problem and to Prof. L. E. Noland and Dr. Samuel Lepkovsky for advice and criticism during the course of the work.

Material and Methods.

The insects studied were the nymphs of *Æshna umbrosa* E. Walker, and were collected during September 1925 and 1926 in Lake Forest Creek, near Madison, Wis. The material used was collected at the same time and from the same place and hence was all of approximately the same age and had been under fairly

uniform environmental conditions. The creek is a spring-fed, woodland stream about two feet wide and varying in depth from two inches to two feet. The stream is choked here and there with water cress to which the nymphs cling. The pH of the water on collecting days was around 8.

When the nymphs were not being used for experimentation, they were kept separately in finger bowls containing lake water, the pH of which ranged around 8.6. A bit of water plant was placed in the bowl with the insect, except in the case of insects subjected to starvation. These were supplied with a pebble on which to cling. The temperature of the water ranged from 20° to 22° C. The nymphs were fed pieces of mealworms three times a week and the water changed an equal number of times. Careful controls were run. When the nymphs were fed and changed to fresh water, the controls were also fed and changed. The nymphs used in the study were of about the same age, usually in the penultimate stage at the start of the experimental work. Three sets of ten insects each were used in each type of experiment and an effort was made to arrange the insects so that the weight of each set was about the same.

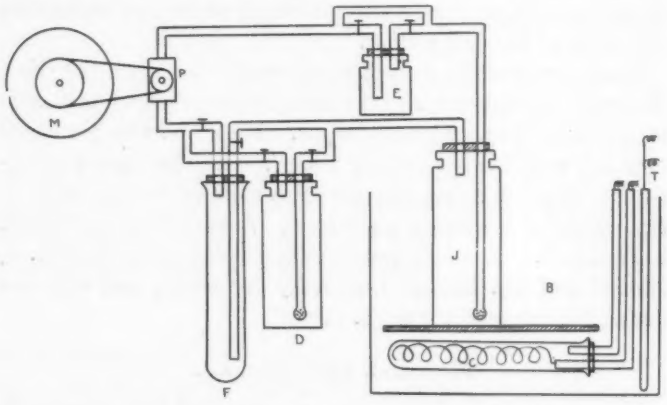


Fig. 1

The experimental apparatus consisted of a closed system of chambers and tubes, through which a circulation of air was maintained by means of a small air pump operated by an electric

motor (Fig. 1, *M* and *P*). The airtight glass jar (*J*) in which the animals were placed, rested in a constant temperature bath (*B*), which was heated by an electric coil (*C*) with thermostat control (*T*). To provide constancy of illumination, light was supplied, when required in the experiment, by a 75-watt electric light bulb which was hung three feet above the jar.

At the beginning of each experiment, 300 cc. of physiological salt solution was placed in the jar. This solution consisted of 7 gr. NaCl, .3 gr. KCl, and .25 gr. $CaCl_2$ per liter of glass distilled water or a total of 7,550 parts per million of dissolved salts. The solution had a specific gravity of 1.003 at 24° C. and a pH near 7.4. Preliminary experiments showed that distilled water, which would naturally be preferred, was quite unsuitable; the nymphs died after a five-hour stay in the water. That the injurious effect of the distilled water was of an osmotic nature was shown by the fact that the addition to the same water of the neutral salts mentioned above made it a satisfactory medium.

In each experiment, a set of ten animals was used and each nymph was placed in a paraffin-dipped wire cage, just large enough to hold the nymph, so that body movement was reduced to a great extent. The cages were closed and tied together in sets of five, with a long thread attached to each set. Experiments were done at the same time each day and always forty-eight hours after feeding, so that excreta had been passed. At the beginning of each experiment, the nymphs were hung above the liquid in the clamp top jar and the threads issued from under the jar cover. The insects were placed in the jar before the CO_2 was removed from the circuit in order to avoid opening the jar after the preparatory run.

After the nymphs were thus arranged in the jar, the motor was started and the air was forced from this jar through a bottle (*D*) containing a solution of sodium hydroxide, which absorbed the carbon dioxide from the circuit and also from the liquid in the experimental jar. A test bottle (*E*) containing a solution of barium hydroxide was then opened into the circuit to determine whether or not all the carbon dioxide had been removed. Fifteen minutes were found to be sufficient time to remove the carbon dioxide. At the end of this time, the clamp top on the experiment

jar was loosened slightly for a moment, releasing the string attached to the cages and allowing them to slip down, immersing the nymphs in the liquid. Bottles *D* and *E* were then cut off from the circuit by the glass stopcock and the experiment was actually started. The air in the circuit was bubbled through the Pyrex test tube (*F*) containing 25 cc. of *N*/20 $Ba(OH)_2$ for five hours. At the end of this period, the clamp top was loosened momentarily and the cages were drawn up to a point above the liquid, and then for fifteen minutes more the air was circulated to remove any carbon dioxide not already absorbed from the water.

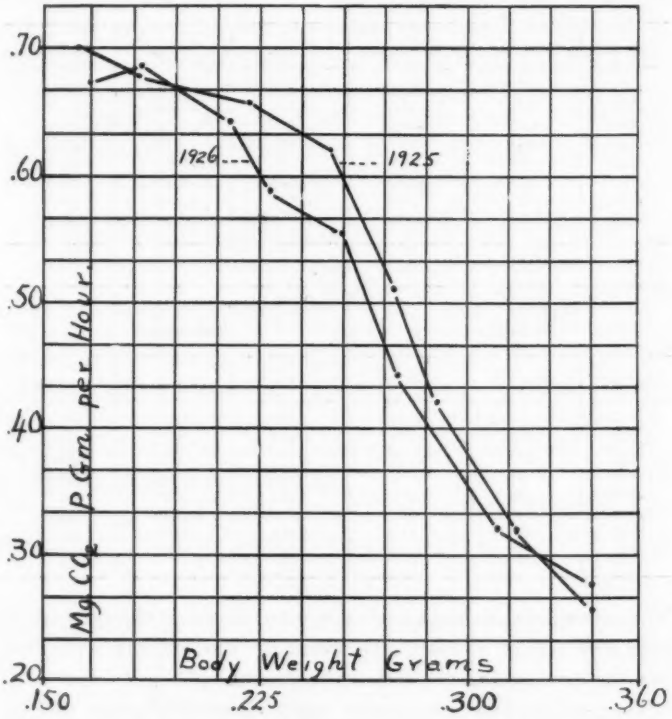
The test tube containing the carbon dioxide precipitated as barium carbonate was detached from the circuit and carefully protected from air until titrated. After the precipitate had settled, the excess of barium hydroxide was titrated with *N*/20 HCl, using phenolphthalein as indicator. To check the accuracy of titration, two aliquot portions (5 cc.) of the clear supernatant liquid were used and the average of the two, multiplied by five, was taken as the true value. This amount was then subtracted from the amount of *N*/20 HCl required to neutralize 25 cc. of the original barium hydroxide solution and the difference was taken as equivalent to the quantity of carbon dioxide produced by the organisms during the period of the experiment. This method of CO_2 determination is based upon the assumption that the only volatile acid produced by the nymphs is carbon dioxide, or that other acids, if produced, are negligible in quantity.

Since the rates of respiratory metabolism are expressed per gram of body weight per unit of time, it was necessary to weigh the insects used in the experiments. Weighing excited the insects greatly, so the process was performed immediately following rather than immediately preceding each experiment. Since the nymphs live in the water and alternately draw water into the rectum and expel it in the respiratory act, it was necessary to remove surplus water from the surface of the body and to see that the nymphs discharged water from the rectum before each weighing. The excess moisture was removed by placing the nymphs between pieces of filter paper. Even though the greatest care was used, it is probable that the amount of moisture remaining in the rectum varied a little at different weighings.

Observations.

A. The Carbon Dioxide Output of Control Insects.

The carbon dioxide output of the nymphs under ordinary laboratory conditions was taken as representing the standard output, to which comparison could be made with the data secured



Graph 1. Curves show rate of CO_2 output over period of 3 months. Time interval 10 days. Temperature 22° C. Abscissas, gram body weight per insect. Ordinates, milligrams CO_2 per gram insect per hour. For further explanation see description in text.

under experimental conditions. The temperature of the water was uniformly between 20° and 22° C. Two sets of insects were used each year (1925 and 1926) and the records of their CO_2 output are given in Table I., and the average record in Graph 1.

TABLE I.

SHOWING THE ACTUAL CO_2 OUTPUT IN MILLIGRAMS PER GRAM BODY WEIGHT PER HOUR, DURING A PERIOD OF THREE MONTHS AT 22° C.
Time interval—10 days.

1925.

Weight of 1 Insect in Grams.			CO_2 Output in Milligrams per Gram Body Weight.		
Set 1.	Set 2.	Average.	Set 1.	Set 2.	Average.
.160	.171	.165	.6630	.7391	.7010
.185	.183	.184	.6950	.6505	.6727
.220	.214	.217	.6501	.6499	.6500
.250	.238	.244	.6402	.5989	.6195
.274	.260	.267	.5218	.5076	.5147
.293	.288	.290	.4129	.4333	.4231
.319	.313	.316	.3343	.3101	.3222
.346	.334	.340	.2697	.2454	.2575

1926.

Weight of 1 Insect in Grams.			CO_2 Output in Milligrams per Gram Body Weight.		
Set 1.	Set 2.	Average.	Set 1.	Set 2.	Average.
.172	.166	.169	.6555	.6900	.6727
.191	.183	.187	.6968	.6731	.6849
.217	.214	.215	.6336	.6504	.6420
.237	.230	.233	.5737	.5903	.5820
.267	.255	.261	.5482	.5545	.5513
.291	.279	.285	.4350	.4791	.4570
.328	.301	.314	.3018	.3508	.3263
.359	.331	.345	.2757	.2884	.2820

The determinations were made every ten days and the data taken over a period of almost three months. The animals were still in good condition at the end of this period.

The results show a relatively smooth curve; the rate of CO_2 output is higher for animals lighter in weight and decreases progressively as the animals increase in body weight. Differences in body weight, especially in nymphs, are closely correlated with differences in age and one is led to assume that younger individuals have the higher rate of respiratory output. Smaller animals are generally more active and are immature and growing.

At 22° C. the saturation point of oxygen at the stated degree of salinity of the chloride solution is about 8. Since each experi-

ment was run over a period of five hours, a determination of the dissolved oxygen content of the water at the beginning and at the end of one of the experiments was made, using the Winkler method as described in "Standard Methods for the Examination of Water and Sewage" (24). The physiological salt solution was found to contain 6.959 parts per million at the start of the experiment and 7.145 parts after five hours. It was thus shown that there was plenty of oxygen in the circuit to meet the needs of the insects for the period of the experiment. The fact that there is more dissolved oxygen in the solution at the end of this experiment than at the beginning is accounted for by the fact that the oxygen of the air in the circuit previous to starting the experiment dissolved in the solution during the five hours of circulation.

B. The Effect of Starvation on the Carbon Dioxide Output.

Three sets of nymphs were used in the starvation experiments, carried on at 22° C. Their carbon dioxide output in a well-fed condition was determined twice at the beginning of the experiment, and then the animals entered starvation. The water was changed regularly, but no food was given. Carbon dioxide determinations were made once a week up to the fifth week. In each set, one or more animals died during the fifth week, which necessarily ended the experiment.

An examination of Table II. and Graph 2 shows that the carbon dioxide output decreased during the first week, but in each

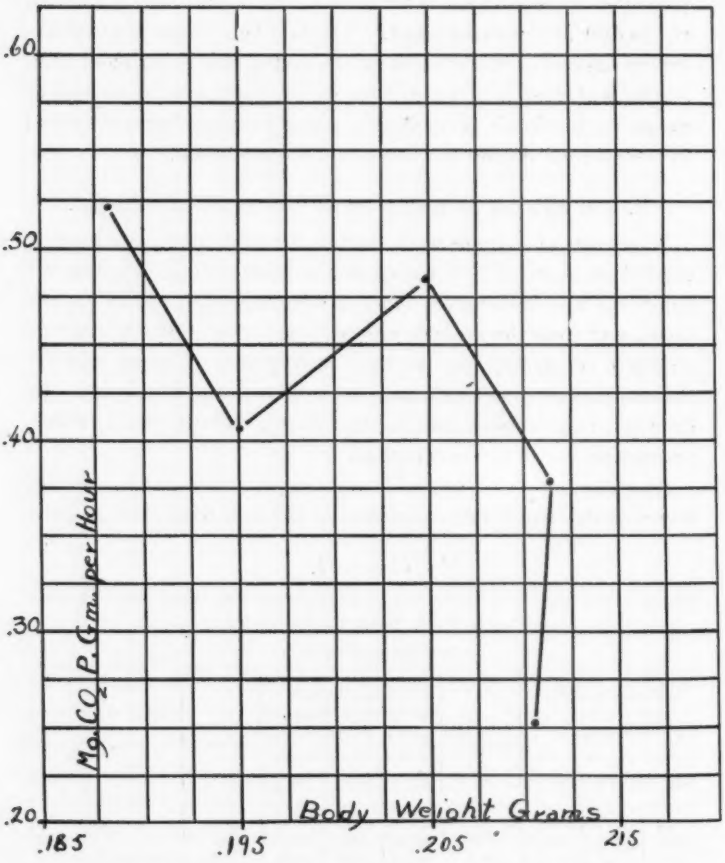
TABLE II.

SHOWING THE ACTUAL CO_2 OUTPUT IN MILLIGRAMS PER GRAM BODY WEIGHT PER HOUR, DURING STARVATION.

Time interval—7 days.

Average Weight of 1 Insect in Grams.			Average Weight.	Milligrams CO_2 Output per Hour per Gram Body Weight.			Average CO_2.	
Set 1.	Set 2.	Set 3.		Set 1.	Set 2.	Set 3.		
.194	.181	.185	.188	.4252	.6077	.5747	.5338	Normal.
.195	.183	.190		.4512	.5809	.5632		
.195	.197	.192	.195	.3333	.4187	.4988	.4169	Starved 1 week.
.194	.220	.201	.205	.4670	.5200	.5073	.4981	Starved 2 weeks.
.199	.231	.205	.212	.4208	.3238	.4033	.3826	Starved 3 weeks.
.200	.229	.205	.211	.2200	.2882	.2671	.2584	Starved 4 weeks.

set the output increased during the second week. The explanation of this increase is questionable. It may be that at this stage the nymph draws heavily on its reserve food or on its tissues. During the third and fourth weeks, the animals showed a steady decrease in metabolic rate. Nearly all the starved nymphs died before the seventh week, although two lived two months without food.



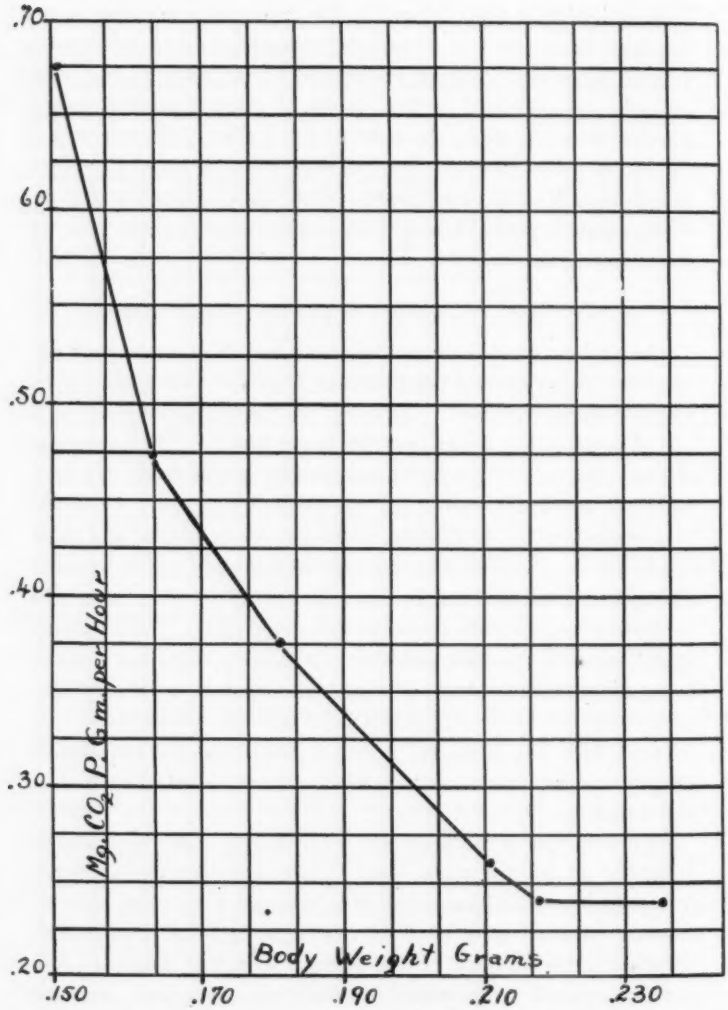
GRAPH 2. Curve shows rate of CO_2 output during four weeks of starvation. Time interval 7 days. Temp. 22° C. Abscissas, gram body weight per insect. Ordinates, milligrams CO_2 per gram insect per hour. For further explanation see description in text.

In comparing this graph with the graph of controls (Graph 1), it is noticed that the carbon dioxide output of a starving insect decreases in one week at an amount comparable to the decrease in twenty days in a younger insect and to ten days in the older animals. At the end of four weeks of starvation, the carbon dioxide output is about the same as the output of the controls at the end of three months. It is interesting also to note an increase in weight in the starving insects. Since the nymphs are aquatic, this increase is probably due to the replacement of reserve fat by water during starvation.

C. The Effect of Darkness on Carbon Dioxide Output.

The carbon dioxide output of three sets of nymphs was determined under normal conditions of light and then the nymphs were placed in darkness. Darkness was maintained by placing a cap of heavy black paper over the finger bowls. The paper was of the type used to protect photographic paper from the light and was admirably suited for the present purpose. Air could pass under the edge of the bag, which was fastened here and there to the table. The temperature remained around 22° C. and the animals were fed as usual, but in a dark room. Sticks were used as the objects upon which the nymphs could cling. Water plants would decay in the dark and offer a possibility of toxic materials being added to the water. During experimentation the room was in darkness and the experimental jar and the water bath were covered with black paper. Once a week the carbon dioxide output was determined and the results are recorded in Table III. and Graph 3.

The metabolic rate decreased progressively with the weeks of darkness up to the sixth week, when four individuals died. Although the experiment was then brought to a close, the remaining nymphs were kept in the dark until all died, which occurred within a period of two weeks. That the metabolic rate of these animals is decreased by darkness can readily be seen by comparing the data here given with the data obtained from the controls. Per unit of weight the CO_2 output of the nymphs was much less than that of the control animals. A control insect weighing .214 gram gave off .6499 milligram of CO_2 per



GRAPH 3. Curve shows rate of CO_2 output in darkness. Time interval 7 days. Temp. 22° C. Abscissas, gram body weight per insect. Ordinates, milligrams CO_2 per gram insect per hour. For further explanation see description in text.

TABLE III.

SHOWING THE ACTUAL CO_2 OUTPUT IN MILLIGRAMS PER GRAM BODY WEIGHT PER HOUR, DURING DARKNESS.

Time interval—7 days.

Average Weight of 1 Insect in Grams.				Milligrams CO_2 per Hour per Gram Body Weight.				
Set 1.	Set 2.	Set 3.	Average.	Set 1.	Set 2.	Set 3.	Average.	
.158	.165	.173	.165	.6613	.7650	.5999	.6754	Light.
.164	.170	.181	.172	.4359	.5931	.4071	.4787	1 week in dark.
.182	.181	.193	.185	.3626	.4207	.3404	.3746	2 weeks in dark.
.211	.199	.207	.206	.2345	.3093	.2303	.2580	3 weeks in dark.
.218	.219	.214	.217	.2523	.2641	.2298	.2487	4 weeks in dark.
.233	.225	.229	.229	.2317	.2207	.2177	.2234	5 weeks in dark.

hour, while an insect in darkness weighing .211 gram produced only .2345 milligram.

Since most of the animals lived but six or seven weeks, the decreased CO_2 production must have been accompanied by a pathological condition resulting in early death. It is evident that light is necessary for the normal physiological processes, and that a lack of it produces an abnormal condition bringing about death. It would be interesting in further research to determine what part of the spectrum is of greatest importance in the normal life of these nymphs.

D. Carbon Dioxide Output with Rising Temperature.

Table IV. gives the actual amounts of CO_2 given off by three sets of insects as the temperature of the water in which they were living was gradually raised. The average figures are shown in Graph 4.

Most of the authors already cited determined the CO_2 output at each increase in temperature without making a study of the metabolic rate after a longer exposure to the same temperature. Instead of making the usual successive determinations at different temperatures, three determinations were made at each temperature. These records were made after twenty-four, forty-eight, and seventy-two hours' exposure to the given temperature. In following this plan, a different curve was secured than is usually drawn. Reference to the graph shows that the CO_2 output goes

up for each temperature after the twenty-four hours of exposure, but goes down again after the longer periods of exposure. The output is about normal after the seventy-two hours at the given temperature. These results were found to be true in the case of each rise in temperature. If one connected with a line the twenty-

TABLE IV.

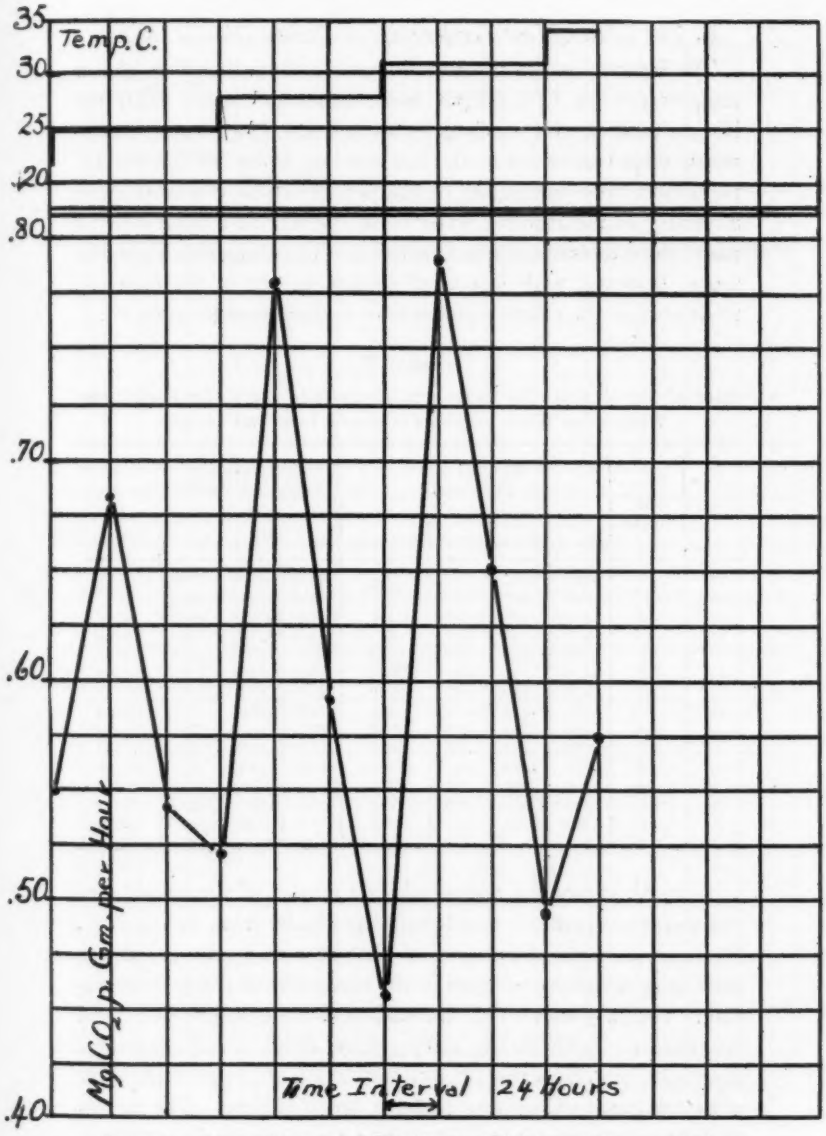
SHOWING THE ACTUAL CO_2 OUTPUT IN MILLIGRAMS PER GRAM TOTAL BODY WEIGHT PER HOUR, DURING PERIODS OF HIGH TEMPERATURE.

Temp.	Time in Hours.	Weight of 1 Insect in Grams.				Milligrams CO_2 per Gram Body Weight per Hour for 1 Insect.			
		Set 1.	Set 2.	Set 3.	Average.	Set 1.	Set 2.	Set 3.	Average.
22		.148	.165	.161	.164	.6087	.5579	.6091	.5499
22		.163	.170	.177		.4723	.5341	.5173	
25	24	.163	.170	.177	.170	.6518	.6742	.7155	.6805
25	48	.163	.173	.177	.171	.4538	.5404	.6496	.5483
25	72	.165	.173	.182	.173	.4320	.5511	.5774	.5202
28	24	.171	.174	.182	.176	.7655	.7915	.7910	.7827
28	48	.171	.177	.182	.177	.4875	.6731	.6101	.5902
28	72	.177	.179	.184	.180	.3946	.5070	.4978	.4665
31	24	.178	.181	.184	.181	.7746	.7999	.7899	.7881
31	48	.178	.181	.184	.181	.6637	.6359	.6449	.6482
31	72	.178	.181	.186	.182	.4400	.5252	.5088	.4913
34	24	.175	.183	.186	.181	.4914	.5100	.6666	.5560

four-hour points on this graph, the usual rising curve could be secured. However, according to the method of determination followed here, the insects show a mechanism for adjustment or acclimatization as evidenced by their carbon dioxide output.

The greatest output is between 28° and 31° C., this representing for the nymphs the maximum in catabolic reactions. Above 31° the temperature has a deleterious effect upon the organism, for the insects respond but slightly to a further rise in temperature. At this high temperature, the decreased carbon dioxide production is no doubt accompanied by a physiological injury, for the animals died after twenty-four hours in water at 34°.

After twenty-four hours at thirty-one degrees, the oxygen content of the water was determined. It was found that the water still contained around 5.9 parts per million.



GRAPH 4. Curves show rate of CO_2 output at high temperatures. Abscissas, time in hours. Ordinates, at top, temperature degrees Centigrade; at bottom, milligrams CO_2 per gram body weight per hour. For further explanation see description in text.

E. The Effect of Low Temperature on Carbon Dioxide Output.

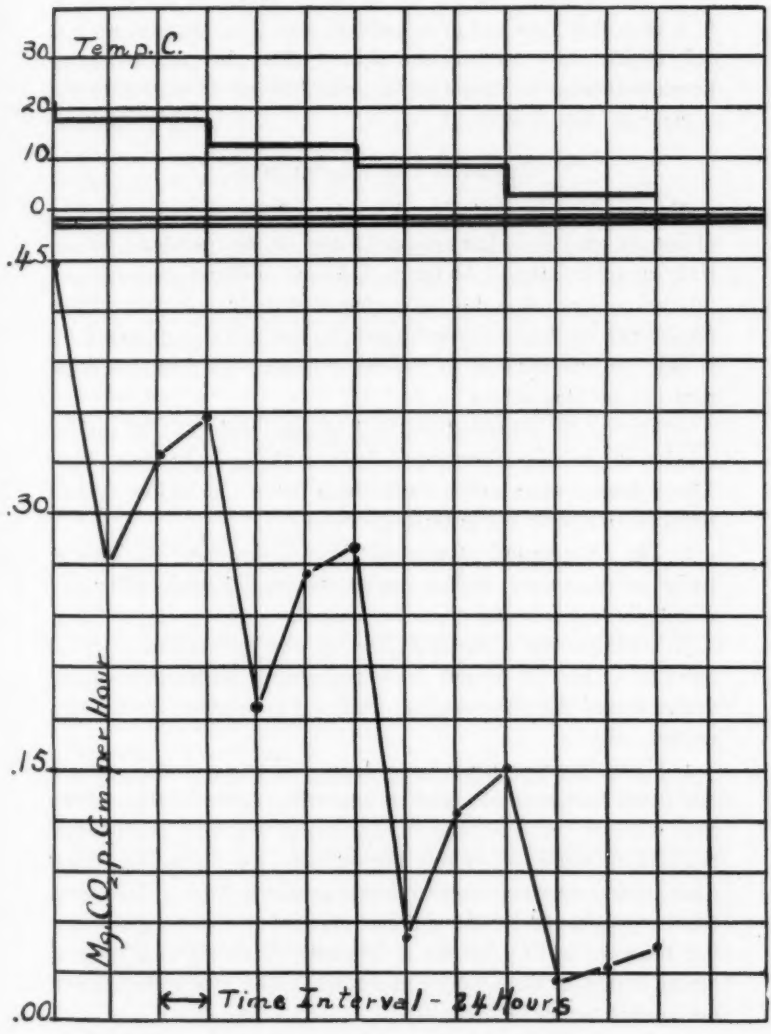
The insects used in the experiments to show the effect of low temperature on CO_2 output were somewhat larger than the insects used in the previous experiments. The former experiments were carried on in the fall months, while the present experiments were carried on in the winter, since it was thought advisable to use running water from the lake at a time when it maintained a constant temperature of 6°. Comparisons may be made, however, with insects of similar weight in the controls, to determine the relative amounts of carbon dioxide given off.

TABLE V.

SHOWING THE ACTUAL CO_2 OUTPUT IN MILLIGRAMS PER GRAM TOTAL BODY WEIGHT PER HOUR, DURING PERIODS OF LOW TEMPERATURE.

Temp.	Time in Hours.	Weight of 1 Insect in Grams.				Milligrams CO_2 per Gram Body Weight per Hour for 1 Insect.			
		Set. 1	Set 2.	Set 3.	Average.	Set 1.	Set 2.	Set 3.	Average.
22°		.258	.243	.211	.240	.4263	.4750	.5001	.4548
22°		.266	.246	.217		.4135	.4519	.4722	
17°	24	.260	.246	.217	.241	.2538	.2891	.2906	.2778
17°	48	.266	.246	.217	.243	.3501	.3477	.3404	.3460
17°	72	.266	.250	.221	.246	.3721	.3655	.3494	.3623
13°	24	.278	.250	.221	.250	.1978	.1861	.2054	.1964
13°	48	.278	.250	.221	.250	.3079	.2737	.2977	.2931
13°	72	.286	.253	.227	.255	.3165	.2999	.3147	.3104
9°	24	.288	.253	.228	.256	.0593	.0577	.0694	.0621
9°	48	.288	.253	.228	.256	.1337	.1272	.1399	.1336
9°	72	.289	.260	.237	.262	.1582	.1488	.1577	.1549
3°	24	.289	.260	.237	.262	.0380	.0299	.0376	.0352
3°	48	.289	.260	.237	.262	.0425	.0471	.0517	.0471
3°	72	.289	.264	.237	.264	.0570	.0441	.0597	.0536

Table V. shows the carbon dioxide output of the nymphs as the water temperature was lowered gradually from 22° to 3° C. The same scheme of duration of time at each temperature was used as previously employed in the records with rising temperature. Graph 5 shows that the carbon dioxide output decreased progressively with falling temperature after twenty-four-hour durations, but also shows an acclimatization to a low temperature after being exposed to it for a longer period of time. The carbon dioxide output drops for each drop in temperature, but the output increases again in forty-eight and seventy-two hours at a



GRAPH 5. Curves show rate of CO_2 output at low temperatures. Abscissas, time in hours. Ordinates, at top, temperature degrees Centigrade; at bottom, milligrams CO_2 per gram body weight per hour. For further explanation see description in text.

given temperature. At 3°, the metabolic processes are going on at a very slow rate and acclimatization at this temperature was only slight. Low temperature did not affect the nymphs in any permanent way, for they lived a normal length of time after the experiment was completed.

Summary and Conclusions.

Although a great deal of work has been done on the metabolism of insects, very little has been published on the metabolic rate in truly aquatic forms. As far as I know, nothing has appeared relative to the carbon dioxide output of Æshnid nymphs. Their respiratory mechanism is an interesting one and the present work is only an introduction to the many related research problems that present themselves.

1. During the nymphal stage of *Æshna umbrosa*, the carbon dioxide output decreases progressively as the nymphs grow older. The younger, more active individuals have the higher rate of respiratory output per gram body weight.

2. The CO_2 output of starved animals decreases during the period of starvation, except during the second week, when the metabolic rate is higher than the normal.

3. Darkness has a marked effect on the metabolism of these animals. The CO_2 output decreases steadily with the weeks in darkness and the physiological condition is so affected, that the nymphs die.

4. Higher temperatures cause increased rates of CO_2 output and lower temperatures tend to have the reverse effect. However, after an initial increase in CO_2 output following twenty-four hours of exposure to the high temperature, the output decreases again after longer exposure to the same temperature. The same effect is evidenced by the nymphs exposed to low temperature, *i.e.*, decrease in CO_2 output is followed by an increase after a longer period at the same temperature. The insects indicate a mechanism for acclimatization.

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FURTHER OBSERVATIONS AND EXPERIMENTS ON THE SYMBIOSIS BETWEEN TERMITES AND THEIR INTESTINAL PROTOZOA.

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It has been possible through a grant from the Bache Fund of the National Academy of Sciences and the collecting ability of Dr. Harold Kirby, Jr., to extend somewhat my observations on the symbiosis between termites and their intestinal protozoa.

Hitherto the investigations (Cleveland, '24, '25*a*, '25*b*) have been confined principally to two genera, *Termopsis* and *Reticulitermes;* similar observations have now been made on six more genera (subgenera according to some investigators), with fragmentary observations on several other genera. All the termites in this investigation except *Reticulitermes* came from Panama and Costa Rica and were identified by Dr. T. E. Snyder.

In most of the experiments the protozoa were removed by confining the termites in oxygen at a pressure of 60 pounds for one hour; in a few instances higher pressures for a shorter period and lower pressures for a longer period were used. It was never possible, regardless of how high a pressure was used, to remove all the protozoa in less than ten minutes. The termites, as in previous experiments (Cleveland, '25*a*, '25*b*), showed no ill effects from the oxygen even if confined in it for a much longer period than that required to kill all their intestinal protozoa.

Large numbers of termites were used when available and after the removal of the protozoa they were kept in large Petri plates and were fed either cellulose (filter paper) or wood. Moisture was supplied in all except the earlier experiments by placing in each Petri plate three small pieces of absorbent cotton dipped in water and then mashed between the fingers until as much water as could be gotten out in this way was removed. Every third

day the pieces of cotton were remoistened. This appears to be the best way to keep termites in the laboratory so that they may be constantly observed with little effort and without being greatly disturbed. When fed wood, they were not given enough to hide themselves by burrowing into it. They prefer the wood and paper and do not eat the cotton unless the supply of wood or paper is exhausted. Death due to molds—the bane of poorly kept laboratory termites—may be avoided entirely when termites are kept in this manner. Controls of all the experiments were kept in the same manner and are in excellent condition now, ten months after the defaunated termites were all dead.

The principal results lend themselves rather easily to tabulation and are set forth in Table I. These data are not as complete as they should be. They show, however, that none of these termites are able to live indefinitely after their protozoa have been removed by oxygen treatment. But, in most cases, they do not show why some termites lived longer than others. This may be a difference inherent in the termites themselves, some being more dependent on protozoa than others. Further investigations, in fact, may even show that certain protozoa-harboring termites are able to live for a long time, if not indefinitely, on a normal diet of wood after their protozoa have been removed. The reason why the termites of some genera lived so much longer than those of other genera may also, in part, be a matter of food. Too few termites were available in some instances to properly control this factor. Some of the termites were strikingly different in appearance and behavior from others, and we may well expect considerable differences in their ability to maintain themselves on the same diet after the removal of their protozoa, but it will be necessary to carry out more extensive experiments before this can be definitely demonstrated. However, a large number of experiments were carried out with *Reticulitermes* under ideal conditions with the result that this termite of the family Rhinotermitidæ was not able, on the average, to live nearly so long as termites of three genera of the family Kalotermitidæ (*Neotermes*, *Kalotermes*, *Cryptotermes*). In the case of *Reticulitermes*, the reason why some of the experiments ran so much longer than others is quite clear. Those experiments which ran so long

TABLE I.

SHOWING APPROXIMATE LENGTH OF LIFE IN DAYS OF VARIOUS DEFAUNATED TERMITES.

Termites.	Number of Experiments Carried Out.	Number of Termites in Each Experiment.	Days before All Termites in One Experiment Were Dead.	Days before All Termites in All Experiments Were Dead.	Average Duration of Experiment or Length of Life of Defaunated Termites.	Food.	Remarks.
Reticulitermes flavipes Kollar.....	20	200	18	68	29	Paper, sound wood, decayed wood.	Kept in Petri plates. Those fed decayed wood lived longest.
Calcaritermes brevicollis Banks ...	5	50	19	29	26	Sound wood.	Kept in jars.
Cryptotermes dudleyi Banks.....	3	50	52	77	66	Sound wood.	Kept in Petri plates.
Lobitermes longicollis Banks	2	200	20	22	21	Sound wood.	Kept in jars.
Neotermes holmgreni Banks.....	4	50	94	123	107	Paper.	Kept in Petri plates.
Glyptotermes barbouri Snyder	3	50	26	28	27	Sound wood.	Kept in large test tubes.
Kalotermes panamæ Snyder.....	2	300	71	80	75	Fairly well-decayed wood.	Kept in Petri plates.
Kalotermes tabogæ Snyder.....	2	50	129	165	147	Paper	Kept in Petri plates. Only a few lived so long.

contained a fairly large number of second form young adults. These individuals are fed and supported by the workers and nymphs (Cleveland, '25*b*). They had few, if any, protozoa to lose (Cleveland, '25*b*) and were able to exist in a fairly normal manner on the salivary secretions of the workers and nymphs for three weeks or more, or as long as the workers and nymphs lived and were able to feed them, and then came their period of starvation followed by death three to four weeks after the workers and nymphs who supported them died.

The conditions under which *Lobitermes*, *Calcaritermes*, and *Glyptotermes* were kept were perhaps not so favorable as the conditions of the other genera of this family (Kalotermitidæ), *Kalotermes*, *Cryptotermes*, and *Neotermes*, and perhaps for this reason they did not live so long. These experiments were carried out before the Petri plate method of keeping termites was discovered, and all termites of these genera were used up.

Some material of two other genera, *Leucotermes* and *Coptotermes*, of the family Rhinotermitidae was procured, but these termites behaved so differently from *Reticulitermes* and the genera of the Kalotermitidæ that it was impossible to carry out the same experiments with them. One or two days in closed jars or test tubes usually resulted in their death. Almost any kind of handling, especially confinement in a closed jar or, sometimes, shipment for eight to ten days in a wooden box, resulted in the loss of their intestinal protozoa. Owing to the fact that material of these genera was not plentiful, no attempt to study them in aërated containers was made. It will be very interesting indeed for comparative purposes, and possibly for gaining some definite information on the origin of termite symbiosis and parasitism, to carry out a large number of experiments on these and other genera of the Rhinotermitidæ. Valuable information in this same direction may also be obtained by a more critical study of the ability of several genera of the Kalotermitidæ to live on various diets after the removal of their protozoa. It is also very important to study the single genus *Mastotermes* of the most primitive family, the Mastotermitidæ.

Several genera of the most highly specialized family, the Termitidæ, were obtained. This family as a rule (Cleveland,

'23*a*, '26) does not harbor protozoa, although a few small flagellates and fairly large xylophagous amœbæ[1] occur in some species, but perhaps not in sufficient numbers, for the most part, to be of any decided advantage to their hosts. Many species in this family do not feed on wood at all, and those that do, feed on more decayed wood than most termites of the other three families. Numerous attempts have been made to feed cellulose (filter paper) to several genera of the Termitidæ (*Anoplotermes*, *Mirotermes*, *Microcerotermes*, *Cornitermes*, *Orthognathotermes*, *Armitermes*, *Convexitermes*), but with no very definite results. These termites eat moistened filter paper readily and it will be an easy matter to determine whether or not they can digest it as soon as a convenient method for keeping them in the laboratory is worked out. In most of the experiments that were carried out the controls, or those termites fed very much decayed wood and fresh soil or humus, lived but little longer than those fed paper. When the termites were fed paper and allowed to remain in the nest or a portion of it, they lived much longer than when placed in jars with paper only. This was perhaps due to the fact that the fungi in the nest aided them in some way in the digestion of food. However, the laboratory conditions at best were too different from those of nature to lend much value to the experiments. When better means of keeping the Termitidæ in the laboratory are obtained, this subject will be considered again.

Termites of all four families harbor very many spirochætes, which, like the protozoa, may play a rôle in the digestion of cellulose and hemicellulose. Considerable time has been given to an attempt to remove the protozoa from several genera of the Kalotermitidæ without removing the spirochætes, but with no very definite results, because practically every treatment that removes the protozoa removes the spirochætes also. It has been possible, however, in a few experiments to remove the protozoa by heat treatment without killing the spirochætes, but they were all dead three or four days later. It will be necessary to repeat these experiments many times under easily controlled conditions before any definite conclusions are drawn regarding the relation of the spirochætes to the termites and to the protozoa. They may

[1] These amœbæ will be described by Dr. Harold Kirby, Jr.

live in some sort of a symbiotic relation to the protozoa—we do not know. They are attached to the protozoa—a fact overlooked by Damon ('26) and by Hollande ('22), who have recently given brief descriptions of termite spirochætes. Very few of the spirochætes, indeed, except those of the large form similar to the *Cristispira* of oysters, are free in the intestinal fluid. Countless millions are often attached to a single protozoön, which seems scarcely able to move. Some protozoa have no attached spirochætes, while others of the same species may have from one individual to several millions attached to them. Sometimes half of the protozoa in a termite are completely covered with spirochætes, which, of course, may be mistaken for flagella unless examined by the dark field method. Spirochætes may also at times be seen inside the protozoa when the termites have been fed paper for a long period. All attempts to grow the spirochætes have failed so far, and all animal inoculations have been negative. The subject is being pursued further and will be reported on in more detail later.

Somewhere in the 1,500 species of termites there is a key to the unique and most interesting animal association throughout all nature. There have probably been many gradatory steps in the formation of the association, but perhaps enough of the steps remain today to enable us, after many years of investigation, to trace the order of events. The fact that in the Termitidæ we find a few protozoa in a species here and there suggests that in this family we are now viewing either the beginning or the end of a widespread symbiotic association. The ease with which those genera of the Rhinotermitidæ resembling the Termitidæ closely in behavior lose their protozoa when handled in the laboratory or when packed and shipped, indicates that possibly the Termitidæ are losing their protozoa.

Addendum.

Since this paper went to press termites have been fed cellulose thoroughly moistened with a five per cent. aqueous solution of acid fuchsin and it has been possible in this way to remove all spirochætes from them without doing any damage whatever to the protozoa or to the termites. Some of the termites have been

spirochæteless now for three months and it is impossible to detect any deviation from their normal appearance and activity, even though they have been fed continuously on the diet which removed their spirochætes. Also no change has occurred in the number and activity of the protozoa. Evidently the spirochætes play little, if any, role in the digestion of wood and cellulose. These results will be reported in detail later.

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NOTES ON A 32 MILLIMETER FREEMARTIN.

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In an earlier paper, Bissonnette ('24) described the development of the reproductive ducts and canals in cattle embryos from 28.0 mm. crown-rump length to 20.0 cm., and compared with that series one of 5 freemartins from 9.0 cm. to 20.0 cm. In that paper the gonad of a 9.0 cm. freemartin was described which showed unmistakable development of ovarian cortex or secondary sexcord region. The material described gave support to the theory of Lillie ('16 and '17) and Keller and Tandler ('16) that the freemartin is a female modified in utero in the male direction by the action of a male hormone from the testis of her twin brother. It showed that she may go so far in the female direction as to have a secondary sexcord region and even to the condition with an intermittent wolffian duct before undergoing regression of the müllerian duct derivatives and added growth and differentiation of the so-called "male" structures. The one there described was the first showing such sexcords. Since earlier writers had agreed that such cords were never found in freemartins (Chapin, '17, Willier, '21, Lillie, '23) the question remained as to whether this was not a very abnormal situation among freemartins. Further study of younger stages of the freemartin was needed to answer this question.

Lillie ('23) described and figured a 3.75 cm. freemartin with no such cords but with a germinal epithelium more than one cell thick—in some places even 3 or 4 cells thick—but never more than about a third to a sixth of the thickness of the corresponding layer in the single-born female. He found the ducts and canals in no way different from those of the female of corresponding size. This was the smallest stage of the freemartin described and it showed definite modification of the gonad in the male direction.

The subject of the study described in this paper is a freemartin

of 32 mm. crown-rump length which is of interest because (1) it is the smallest stage so far studied, (2) it has a minimum of union of the chorions compatible with any blood transfusion, (3) it is another case where ovarian cortex is found outside the tunica albuginea, (4) it appears to be as near as possible to the stage of distinguishable sex-differentiation in which modifications of the freemartin type can be distinguished, (5) it shows slight freemartin modification and forms the initial end of a series of intergrades between the postnatal freemartin types and the normal female.

Material and Methods.

This 32 mm. freemartin, twin to a 34 mm. male, was taken July 28, 1923, at the killing floor of Swift & Co., Chicago Stockyards. Since it was the smallest freemartin in over 50 taken up to that time by the writer, it was especially carefully treated. The uterus of the mother was slit open throughout both horns and the membranes and twins carefully lifted out. The embryos were far up the opposite horns of the uterus and the chorions were but slightly united and showed no bloodvessel union visible to the naked eye. The placentæ pulled apart with a small amount of bleeding only, when they were handled after removal, though they held together during that operation. Chorions and embryos entire were placed at once into Bouin's fluid which was changed once about two hours after they were put in. No injection was attempted since the vessels were judged too small for the apparatus at hand on the killing floor and the help available, and the internal organs were wanted for microscopic work and early fixation was desired. The membranes and embryos were washed in 50 and 70 per cent. alcohols, to the latter of which at its second addition a few drops of a saturated aqueous solution of lithium carbonate was added to help remove the picric acid. They were then stored in 80 per cent. alcohol for about a year and a half, when the embryos were removed and the posterior parts of both embryos were stained in bulk in alum cochineal and sectioned 30 micra thick. The series was counterstained on the slide in orange G and mounted in balsam.

Both maternal ovaries had been taken by the boy who collects those with large corpora lutea for extraction, so it is supposed each had a corpus luteum.

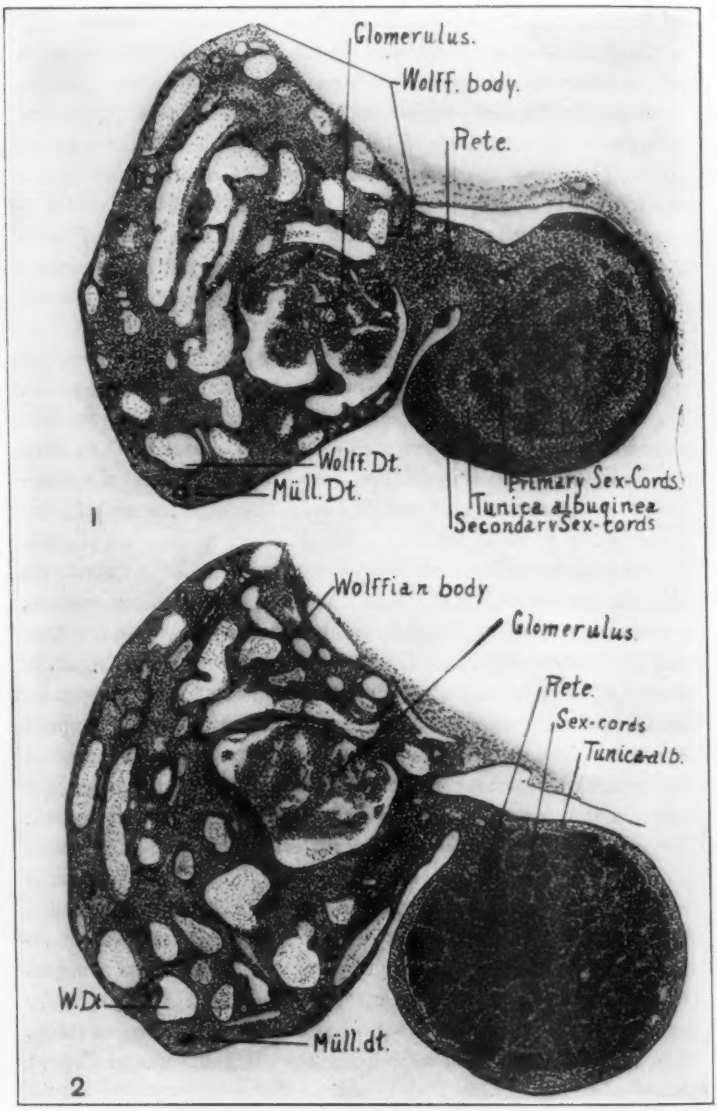

FIG. 1. Transverse section through gonad, mesonephros, and ducts of right side of normal female, 32 mm. crown-rump length. × 38.3.

FIG. 2. Transverse section through right gonad, mesonephros, and ducts of normal male, 32 mm. crown-rump length. × 38.3.

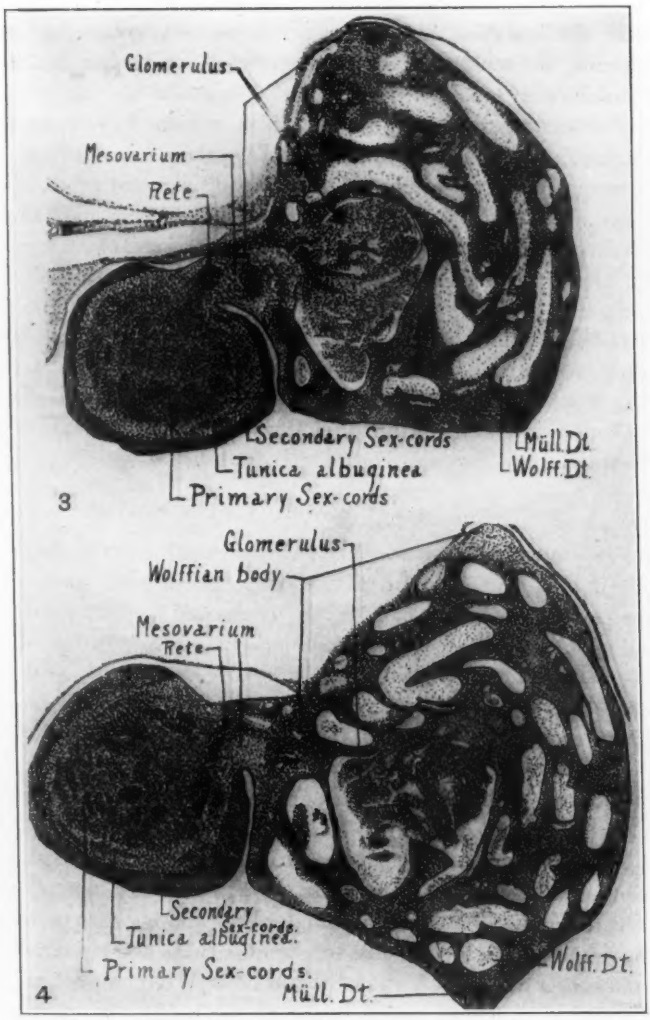

FIG. 3. Transverse section through left gonad, mesonephros, and ducts of freemartin, 32 mm. crown-rump length. × 34.3.

FIG. 4. Transverse section through left gonad, mesonephros, and ducts of normal female, 32 mm. crown-rump length. × 34.3.

On washing out, no vascular anastomosis could be made out with the binocular dissecting microscope, low power, but the bleeding on separation was evidence that there was capillary connection at least.

Previous work had shown that thick sections have some advantages over thin in showing the zones in the gonads and regions of even slight degeneration with consequent pigment production. They also save time in reconstruction of the ducts and canals. Such reconstruction was made for this freemartin and for a normal female of the same size for a control, after the manner of Bissonnette ('24). The notochord was used as reference line for dorso-ventral relations of ducts, canals, mesonephros, and anlagen of the round ligament or gubernaculum, and gonads (Figs. 5 and 6). Typical cross-sections through the gonad, mesonephros, müllerian and wolffian ducts, in the normal female, male, and freemartin, are shown in Figs. 1, 2, 3, and 4.

Description and Comparison with Normals.

The Placental Situation.—The twins were far apart in discrete but united chorions with cotyledons beginning to appear as opaque patches. One chorion was telescoped slightly into the end of the other and they were so united that bleeding occurred on separation. Otherwise the chorions were normal. Both older and younger cases have been taken by the writer in which the chorions were in contact but not so adherent as to cause bleeding on separation. In all such cases so far investigated the female has proved normal. This situation was much the same as that in the 9.0 cm. freemartin No. 1, Fig. 2, of Bissonnette's ('24) paper, where a slight though definite amount of modification in the male direction was demonstrated and secondary sexcord regions or cords of Pflüger were found. It is another case permitting a small blood interchange between the twins and a relatively small concentration of hormone would be expected on the female side and so a longer period might elapse before the effective minimum concentration of the hormone is reached. The amnions were separated widely and were normal.

The External Genitalia.—Externally this freemartin was female in type as shown by Figs. 5 and 6 in sagittal section. The

clitoris was slightly more recurved than that of the normal control. However there are variations in this respect among single females due to the difference in relative curvatures of the embryos at the time of killing and fixing. It resembles the one figured

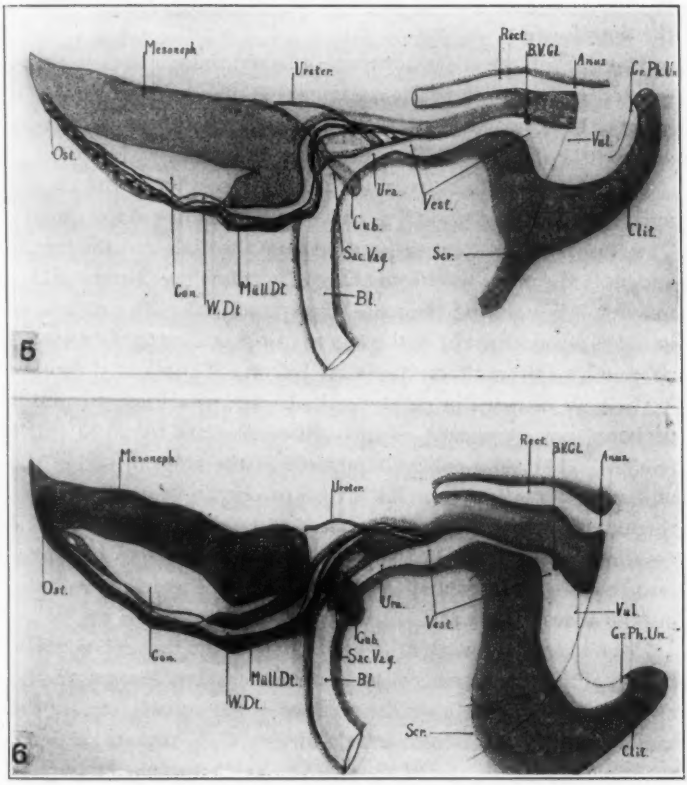

FIG. 5. Reconstruction of the urinogenital system of the freemartin of 32 mm. crown-rump length. × 9.5.

FIG. 6. Reconstruction of the urinogenital system of the normal female of 32 mm. crown-rump length. × 9.5.

by Lillie ('23) for his 3.75 cm. one (Fig. 5) externally as well as in gross internal dissection. So it is not figured here in these aspects. The urogenital aperture is open although in the male of the same size and in the male twin it is closed and has a raphe

to mark the midline in place of a slit. The perineum is not expanded ventrally as in the male.

The vulva, vestibule, and bulbo-vestibular glands are female in type and proportions (Figs. 5 and 6). The ureters also come off the urethra or urogenital sinus as in the female from a ridge in the dorsal wall.

The wolffian ducts are not much affected though their lumen is greater just posterior to the mesonephros than in the control. It is not to be expected that they would be affected greatly since they are still the ducts of the functional mesonephros.

The müllerian ducts are not so large in section as the control and lack a lumen in some regions where there is one in the control. They appear to be intermediate between the male and the female and, as in the male, show some signs of regression. Their staining reaction is like that of the male (Figs. 1, 2, 3, 4). This difference in size is most apparent in the region just posterior to the mesonephros where the wolffian ducts are larger. The slight difference in the type of entry into the vestibule shown in Figs. 5 and 6 is probably not significant as such differences are found in single females. The ostia tubæ abdominale at the anterior end of the müllerian ducts open out flat with lips on the ventral aspect of the anterior end of the mesonephros and show a tendency to be associated with the rete there. This rete appears to lead over into the mesonephros and to join a glomerulus as in the normals of both sexes at this age.

The Gonads.—Figs. 1 and 2 are from sections through near the middle of the right gonads of 32 mm. female and male respectively, and figures 5 and 6 are similar sections of left gonads, etc., of the same sized freemartin and female, drawn with camera lucida to the same scale. As stated above, these thick sections (25 and 30 micra) show up the regions of the gonad better than thin sections, and cortical regions stand out better. The gonads of the freemartin have the same relations to the mesonephros as those of the female. Rete ovarii are similar in that they do not extend into the middle of the gonad as they do in the male, but are found only near the mesenterial border of the gland (Willier, '21; Bissonnette, '24). The type of tunica albuginea is female but the medulla of the ovary does not show signs of as great activity

as in the control. A cortex is present outside the tunica albuginea and is continuous with the germinal epithelium or serosa coat from which it appears to develop, though organization into definite sexcords is not evident in the preparations of either female or freemartin. The secondary sexcord region is not quite so thick as in the control nor is the gonad quite so large in cross-section. It is about 21/23 of the normal in diameter. The length of the freemartin gonad is 1,740 micra while that of the control is 2,150. This may or may not be within the limits of normal variation at this age for no series of measurements of normal ovaries has been made at this stage to determine these limits. The difference in size is apparent from the sections drawn to the same scale. This would be even greater were both sections taken from exactly the same relative level of the gonad. The freemartin was cut 62/100 of the length from the front end and the female 58/100 of the corresponding distance. As shown in the reconstructions, the gonads are larger in diameter posterior to their middle regions but near them. The blood supply of the freemartin and control gonads is distributed through the tunica albuginea inside the cortex, while, in the corresponding male, since there is no cortex outside the tunica, the blood supply is superficial in the definitive tunica albuginea, present at this stage. In the testis at this stage there is marked cellular growth in the medulla or primary sexcord region; in the ovary this activity is less marked; and even less so in the freemartin gonad.

The anlagen of the gubernacula or round ligaments are similar to those of both sexes at this stage. No sex differences in this organ appear till later stages as described by Bissonnette ('24).

Discussion.

Lillie ('16 and '17) and Keller and Tandler ('16) came to the conclusion that the freemartin is a modified female, that sex-determination as a female was consummated at fertilization, and that the abnormalities are due to the action of a male hormone passed to her through the vascular anastomosis in the united placental circulations. They believed that this was a case of normal zygotic sex-determination followed by abnormal sex-differentiation in the female twin. This material supports such

a theory by showing a case near the initiation of the differentiation process where the embryo is essentially female in type and condition of the organs, but is slightly underdeveloped in respect to the secondary sexcord region and müllerian ducts. This case, taken in connection with other described cases, particularly where the placentæ were in contact but not united by either capillaries or arteries or veins, as described by Lillie ('17) and Keller and Tandler ('16) and verified by the writer, appears to show that a blood-carried agent or hormone is at work here and not some agent like the "organizer" which Spemann ('25) shows so unquestionably is operative in the early development of the amphibian larva. If such an agent were operating here, mere tissue cohesion or connection would be sufficient to condition full effect, and the great differences between freemartins of the same embryonic age would be difficult to account for except on the basis of variations in zygotic sex-balance of the two individuals of the twins or differences in time of contact of the chorions. Now, in cattle, mere contact is usually attained early by reason of the great elongation of the trophoblastic vesicle; but vascular connection is not always so early, and the effect appears to depend on blood transfusion rather than on tissue continuity.

However, this case and others such as the one described by Keller and Tandler ('16), in which there was slight vascular interchange accompanied by a scarlike welt across the place of junction of the chorions, reducing what they believed was once a greater vascular connection to proportions too small to account for the degree of modification, appear to show that even a capillary anastomosis or an anastomosis of very small bloodvessels is sufficient to condition the modification. The state of the organs appears to depend on the time elapsed since the effective threshold of hormone action is reached, rather than on a varying amount of effective hormone above such threshold. This freemartin and the 9.0 cm. one of Bissonnette ('24) both showed a minimum vascular connection accompanied by a type of modification, or degree of it, which suggests a marked development as a normal female, probably for some time before the hormone became active, followed by regression of the peculiarly female organs and a delayed acceleration of the growth of the essentially

male parts. They do not support the assumption that there has been a slower than normal development of the female structures with a slightly faster development of the male ones over the whole period of union, as will appear from what follows.

Lillie's Fig. 11, page 68, of a gonad section from a 3.75 cm. freemartin, in which he found demonstrable vessels connecting the circulations of the twins with no clearly distinguishable place of chorionic union, shows a germinal epithelium together with three or even four layers of cells outside the tunica albuginea in some regions. These cells of the extra layers are irregularly arranged and not in definite cords. These extra cell layers over and above the single layer of cœlomic epithelium homologous to that in the bull of similar size (Fig. 12) he does not interpret as a sexcord region and cords are not possible with so few cells in any case. After inspection of the 32 mm. freemartin in this study and all the freemartins younger than the 9.0 cm. stages examined by the writer microscopically, all of which show the secondary sexcord region, it appears that these extra cells in Lillie's specimen may well be secondary sexcord primordia in which cords have not yet organized. Brambell ('27) finds that in the mouse cords do not organize till later and that in males the germinal epithelium becomes several cell-layers thick after the tunica albuginea forms but later thins out to a single layer. This latter situation does not develop in males in cattle as seen in Fig. 2 of this paper and Fig. 12 of Lillie ('23). Chapin ('17) in some of her gonad sections shows regions which she does not interpret as derivatives from secondary sexcord anlagen, but which appear in the light of this more recent younger material and from an inspection of her slides themselves (kindly loaned by Professor F. R. Lillie) to be derived from such source with possible later proliferation. In any case the secondary sexcord region is not a layer of uniform thickness all over the gonad but is thicker in lateral regions than in ventral. The layer also appears to be resorbed at different rates in different regions and so to remain in some regions longer than in others and possibly permanently. This appears to be correlated with or dependent on pressure by adjacent organs in some way. It is possible for the tunica albuginea to reach the outside of the gonad in some regions and not in others, leaving bands of secondary

sexcord derivatives extending lengthwise in the gonad parallel to the medullary cord derivatives. This situation is figured by other workers. These remnants were difficult or impossible to interpret without comparison with the earlier stages or with stages which had undoubted secondary sexcords. Such a series was not available for study by other students of the freemartin. This will be discussed more fully in a later paper on the basis of more material.

From a study of the above mentioned freemartins and of a 6.75 cm. and 7.5 cm. pair from a set of triplets with one bull only (to be discussed in a later paper) where cords of Pflüger are also present but show signs of regression, one is led to conclude that the rate of resorption of these cords is comparatively slow as well as that they may continue to grow in some cases for a short time before regressing. This also leads one to believe that secondary sexcord regions were never present in Lillie's specimen mentioned above, in any greater amount than is shown in his figure (Lillie, '23, Fig. 11, p. 68). This also suggests that the initiation of the male hormone action as inhibitor of the secondary sexcord region was longer delayed in this 32 mm. one than in Lillie's 3.75 cm. one. This may be explained on the basis of the small amount of hormone passing to the female at first through the small vascular connection. This would delay the effective minimum concentration of the hormone. That there is such an effective minimum, in some mammals at least, is shown by the partial castration and grafting experiments of Moore ('21), Sand ('19), Steinach ('20), and many others for postnatal stages. No prenatal testis grafts have yet been possible in mammals so far as the writer can learn.

It is possible that the vascular anastomosis was just becoming established in these twins and that, as the pregnancy progressed, it would have become more effective by the pressing together of the chorions. This would insure a continuous larger supply of hormone on the female side and the rate of modification in this case would be as fast as in those with earlier complete anastomosis, though the condition reached at any time would lag behind that of the others and this freemartin would fall in the group showing small degree of modification as classified by Willier ('21).

Willier ('21) classifies freemartins into three classes on the

basis of degree of modification—those with small, medium, and large amount of abnormality—and Lillie states that the freemartins "form an exceedingly well-defined group without intergrades to normal females or normal males." This may be true for postnatal freemartins; but, from the series discussed in this and other papers cited, it appears that a series of intergrades connects the female with the freemartin group, or that the freemartin group moves steadily away from the female group of types, as we pass from younger to older stages. No such series is seen between the freemartin and male. The rare cases of modification externally in the male direction are probably due to upset of some kind in genetic or zygotic sex balance and not to the freemartin type of hormonic modification alone. Such cases occur without twinning in cattle, and in as great a percentage of cases as in freemartins. It is this series in the younger stages leading back to the normal female type among freemartins that so conclusively shows that the freemartin is a modified female and not a modified male. Taken with Lillie's ('17) statistics as to sex-ratios in cattle twins, it furnishes most conclusive proof of the theory of the freemartin of Lillie and of Keller and Tandler.

Lillie ('23) suggests that probably there are individual differences in zygotic balance of sex factors in cattle even in single born calves, and those who have been much associated with cattle breeding and judging, and have seen the differences in potency of males and females inter se, and their differing degrees of male and female "conformation," will agree. This appears to be a fundamental factor in the condition of all freemartins and doubtless works from both sides, male as well as female, in the pair. The hormone, even, may not be equally potent in all cases. Its appearance may be delayed in some males for this reason. So the initial action may be delayed even in cases with early complete intercommunication of blood vessels. The females also may differ in their resistance to the masculinizing action of the male hormone: they may differ already in femaleness. Comparison between this case and Lillie's and other small freemartins illustrates how these factors may lead to variations, among the early stages at least. It is also just possible that the differences from the control female found in this specimen may be

within the limits of individual variation resulting from these differences in zygotic sex balance. Only a careful study by biometric methods of a large number of single female embryos at this stage can settle this point. Such a study has not been made because of lack of material and the long time involved in search.

In any case, should this case fall inside the limits of individual variation discussed in the preceding paragraph, it will show all the more conclusively that the first stage of the freemartin is, at least, in some cases, a female, normal for that stage of development.

Conclusions.

1. On the basis of this 32 mm. freemartin and comparison with others described by the writer and by others, where the freemartin modifications are conditioned by vascular unions between the placentæ and not by mere contact and adhesion of one to the other, it is concluded that a blood-carried agent in the nature of a hormone is effective in producing the freemartin rather than an "organizer" of the type found in Spemann's work on early amphibian development.

2. Small vascular or capillary anastomosis is sufficient to condition the freemartin effects in some cases at least; but such small amounts of transfusion as are possible in such cases at first appear to delay the modifications and permit longer female development before the effective minimal concentration of hormone is reached on the female side. Early stages in such cases show a more markedly female embryo than do those with strong anastomosis, particularly in respect to gonad and müllerian ducts. Study of such borderline cases as these lend support to the "all-or-none" conception of the hormone action.

3. Cords of Pflüger or secondary sexcord regions develop frequently in freemartins and are resorbed later at unequal rates in different parts of the gonad, so that some of the peculiarly complicated interiors of gonads figured by previous students of the freemartin gonad can be interpreted in the light of these earlier stages as the results of survivals of isolated parts of the secondary sexcord region, separated from the medulla of the gland by tunica albuginea. Organization into sexcords may not be possible from the first and some individuals may not develop

cords though they may have the cell proliferations from which the cords would later develop but for the action of the hormone. This unequal development of sexcord region and unequal resorption seem to be related in some way to pressure by surrounding organs.

4. There is no intergrading in freemartins between the freemartin types and male types except in rare cases which resemble the anomalies found among single born males, where the sex balance appears to be upset. And in postnatal freemartins there is no intergrading to the female type. But in prenatal freemartins there is a definite series of intergrades to the female condition as the younger stages are studied till practically the female condition is reached. This furnishes the strongest of proof for the theory of the freemartin maintained by Lillie and by Keller and Tandler.

5. There are probably individual differences in zygotic sex balance among both male and female cattle. This is a factor in the variations of condition among freemartins and doubtless is operative from the bull's side as well as on the freemartin side. The hormone may not be equally potent in all cases and it may possibly differ in potency at different times in the same male, or be delayed in its appearance. In this way the hormone action may be delayed even when early complete anastomosis is effected. So, too, it is possible that the differences between this freemartin and the control female may be within the limits of individual variation correlated with zygotic differences in sex balance, for the exact limits of such variation have not yet been determined.

6. If so, it is even stronger proof for the theory of the freemartin of Lillie and of Keller and Tandler, since it would show that the first stage of the freemartin is a female, normal in sexual condition for that age.

Summary.

1. A 32 mm. freemartin twin to a 34 mm. male is compared with a normal female of like size as to reproductive organs, and with normal male as to gonad.

2. It differs from the female control in having a thinner secondary sexcord region and thinner müllerian duct, lacking lumen in places where the control has one. Nevertheless it is more like

the female than any other freemartins with vascular anastomosis so far studied.

3. Placental vascular anastomosis between the twins was very slight—hardly more than capillary—and the placentæ pulled apart with slight bleeding. So it permitted very limited transfusion of male hormone, a situation correlated with slight modification from female type shown in small gonad and thin ovarian cortex and müllerian duct.

4. Comparison with other freemartins as to placental and sexual conditions indicates a blood-borne hormone as the agent rather than an "organizer" found effective in early amphibian differentiation. Mere tissue cohesion fails to condition the modifications as it does in Spemann's transplants.

5. Comparison with other gonads figured for freemartins and with new material indicates that some formerly anomalous regions in older freemartin gonads are derived from secondary sexcord remnants which failed to be resorbed and that these cortical regions frequently develop in freemartins to some degree and may or may not disappear.

6. This study adds support to the theory of the freemartin of Lillie and Keller and Tandler, that it is female, normally determined zygotically, but abnormally differentiated sexually by the intervention of a male hormone received from her twin brother in utero.

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ABBREVIATIONS.

Bl., bladder.
B. V. Gl., bulbovestibular or Bartholin's glands.
Clit., clitoris.
Gon., gonad.
Gr. Ph. Un., undercut groove in phallus.
Gub., gubernaculum.
Mesoneph., mesonephros.
Mül. Dt., müllerian duct.
Ost., ostium tubæ abdominale.
Rect., rectum.
Sac. Vag., saccus vaginalis.
Scr., scrotum.
Ura., urethra.
Vest., vestibule.
Vul., vulva.
W. Dt., Wolffian duct.

THE DIGESTIVE SYSTEM AND ITS FUNCTION IN *FUNDULUS HETEROCLITUS.*

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In the course of investigation of the function of the alimentary canal in *Fundulus heteroclitus* (North American common killifish, mudfish, mummichog [1]), it was found that this animal does not possess a stomach, *i.e.*, an organ secreting the pepsin-hydrochloric acid. Although several species of fishes do not possess peptic glands (see in Oppel's "Lehrbuch der Vergleichenden Mikroskopischen Anatomie der Wirbeltiere," 1 Teil, Jena 1896, S. 33, the corresponding table), we have never seen any reference to any investigation concerning the gastric digestion in *Fundulus heteroclitus*.[1] From the anatomical as well as from the physiological point of view, the digestive system of the *Fundulus* presents great peculiarities, which are worth attention. The present paper is to be regarded as a preliminary communication. A series of investigations were made by one of us (B. P. B.) during the summer of 1926 at the Atlantic Biological Station, St. Andrews, N. B., and subsequently one of us (D. J. B.) made histological examinations at the Department of Physiology, University of Toronto.

Anatomical Data.

The whole family of Pœciliidæ are comparatively small fishes. The largest specimens of *Fundulus* taken at Birch Cove on the Bay of Fundy, near St. Andrews, N. B., were from 65 to 83 mm.

[1] It is interesting to note that the whole family of Cyprinidæ (*Cyprinus carpio*, etc.) are deprived of peptic glands. *Fundulus* belongs to the family of Cyprinodontidæ, and the Germans call it "Amerikanische Zahnkarpfen" (*Brunning* (2)). According to *Gill* (3) "the Cyprinodonts or Poecilids are really related to the Esocids and Umbrids, and to them they should be approximated in the sub-order Haplomi." The results of the present investigation, especially the absence of the glands secreting the pepsin-hydrochloric acid, show that *Fundulus* has some features in common with the Cyprinidæ.

in length, measured from the snout to the insertion of the caudal fin.

The alimentary canal in this species is comparatively short, being about equal to the length of the body, excluding the caudal fin. On opening the abdomen and carefully dissecting away the liver, we see a short œsophagus directly connected to the intestine.

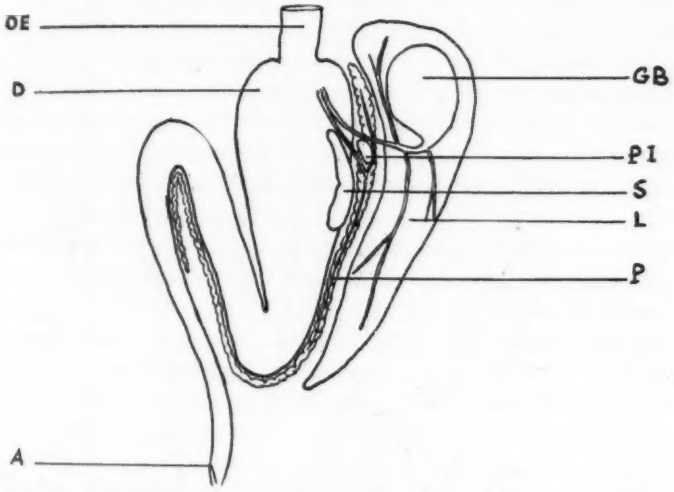

FIG. 1. Diagram of the alimentary tract of *Fundulus*. *Œ*, œsophagus; *D*, duodenum; *GB*, gall-bladder; *P.I.*, principal islands of Langerhans; *S*, spleen; *L*, liver; *P*, pancreas; *A*, anus.

Longitudinal sections through the junction of œsophagus and intestine show that, at this point, there is a well-developed sphincter quite comparable to the pyloric sphincter in mammals. The intestine is bent upon itself ventrally and to the right to form three portions; a first portion, descending; a second portion, ascending; and a third portion, descending to the rectum (Fig. 1). Pyloric cæcæ are not present.

The first part of the intestine, especially when distended with food, has a somewhat pear-shaped form. The second and third parts of the intestine do not display any such unusual degree of dilatation when food is present in them.

In Fig. 2 is represented a scheme of the alimentary tract of

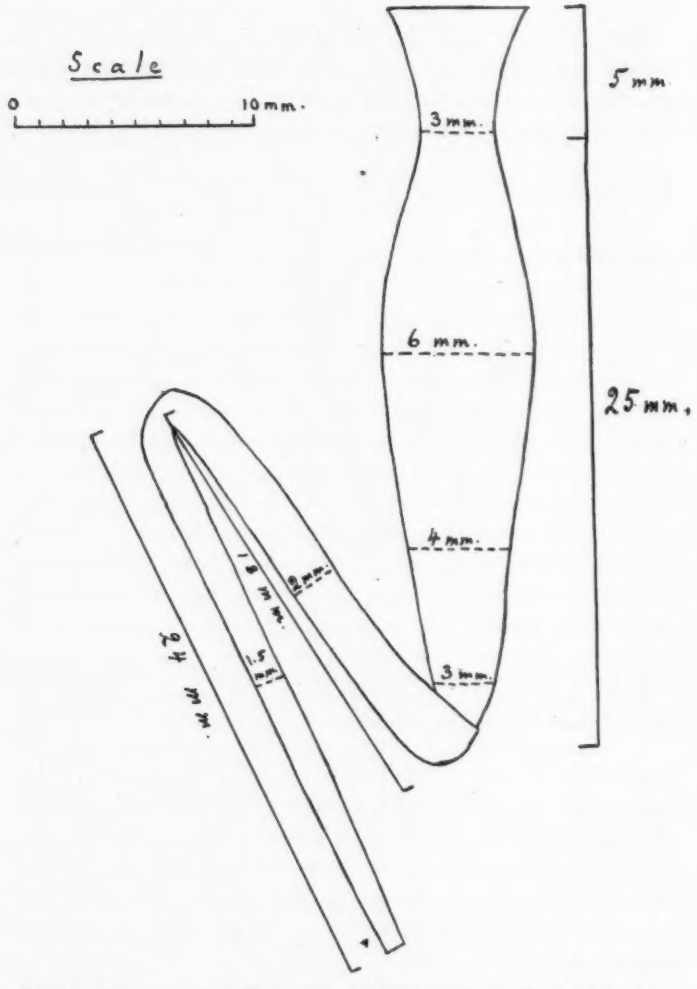

FIG. 2. Scheme of the alimentary tract of *Fundulus*. The intestinal tract is fully relaxed.

Fundulus. The actual measurements are taken from a female *Fundulus* whose body length (caudal fin excluded) was equal to 78 mm.

The capacity of the first part of the intestine is four times greater than the capacity of the second part. Without pressure in the first part, four drops of water enter; in the second part only one drop enters. Under a moderate pressure (filling from a pipette) the cardiac part retains 7 drops, and 8 drops more may be introduced into it by using a greater pressure. Thus the arrangement of the initial part of the alimentary canal of *Fundulus* is similar to the so-called "syphonal" stomach of many other fishes. From a physiological point of view the first part of the intestine of the *Fundulus* fulfils one of the duties of the stomach, being *a receptacle and container for the food*.

In order to avoid repetition of cumbersome phraseology we shall frequently designate the first part of the intestine as the duodenum, since, as will be shown, it is into this part that the bile and pancreatic juice are poured. Both the physiological and the anatomical observations show that a stomach is really absent in *Fundulus*.

The liver is comparatively large, as it is in most fishes, and lies in a left ventral position, closely applied to the œsophagus and to the loop formed by the first and second portions of the intestine. In the upper part of the longitudinal space bounded by the liver and the three portions of the intestine, we find the gall-bladder, the spleen and the "principal" islets of Langerhans, along with a considerable portion of the diffuse pancreas. The pancreas has a wide distribution along the three parts of the intestine and is so diffuse that in fresh specimens it cannot readily be differentiated with the naked eye. The gall-bladder is comparatively large in these fish; in a specimen 78 mm. in body-length it measured 6 mm. by 3.5 mm., and had a capacity of 2.5 drops. The duct which drains the gall-bladder arises from the posterior end of that vesicle, but soon turns in an anterior direction and, after passing along the first part of the intestine to within 3 or 4 mm. of the œsophagus, it pierces the intestinal wall.

It is important to note that the pancreatic duct, though it runs parallel to the bile duct, does not unite with the latter to form a common duct, but has its own separate opening into the intestine

beside the orifice of the bile channel. The hepatic ducts communicate with the cystic duct to form the common bile duct, though one or more of them may empty directly into the neck of the gall-bladder. The spleen is a small, flattened, bright red organ, and serves as a convenient landmark for locating the so-called principal islets of Langerhans, which are situated in the region between the neck of the gall-bladder and the duodenum. The largest of them, the "chief" principal islet, appears as a whitish, spherical nodule about 1 mm. in diameter. The pancreas is best studied in a series of stained sections. These show that slender processes extend off from the larger portions of the gland to invade the liver, particularly along the blood vessels and less so along the hepatic ducts. Sections show also that a thin layer of pancreatic tissue completely envelopes the gall-bladder. Serial sections made with the aim to study the duct connections of the pancreas which surrounds the gall-bladder showed that the ducts are leading away from this pancreatic tissue and communicating with the larger pancreatic ducts. It is important to note, however, that in no case were we able to find any evidence of communication between any of the channels for bile and any of those for pancreatic juice.

Methods.

Since the fistula method is not practical for the study of the digestive processes in fishes, after a suitable interval following natural or forced feeding the contents of the duodenum were sucked out by means of a pipette with a rubber bulb on the end. This method had the well known disadvantage that the intestinal secretions were mixed with food masses and also that removal of the partially digested food caused more or less disturbance of the normal process of digestion. *Funduli* are exceptionally hardy fishes and did not show any ill effects from the manipulations connected with the investigations of their intestinal contents.

The fish were kept in small aquaria or in glass jars containing running sea-water, or else in jars in which the water was kept cool (below 15° C.) and was well aërated. When kept in non-aërated sea-water which is allowed to reach a temperature of 15° to 20° C. (a comparatively high temperature for these *Funduli*), the fish very often eject the food through the mouth and some of them may die under such unfavorable conditions.

The reaction of the intestinal contents was first tested by means of litmus paper. Since the indications of litmus are not very reliable, whenever possible the hydrogen ion concentration was determined by the "spot" method elaborated by Felton (4). The values of the pH found in the present investigation must be looked upon as approximate only, because in most cases the intestinal fluids were mixed with food masses, a fact which would introduce the "protein" and "salt" errors described by Clark (5). The presence of bile in the intestinal contents was determined by Gmelin's test and by the surface tension test with flowers of sulphur. The methods employed for the detection of different enzymatic actions will be given later on.

As a preliminary measure all of the fish were deprived of food for one or more days prior to the experiments.

Experiments on Fasting *Funduli*.

Usually in fishes which have fasted for one or more days the duodenum is practically empty, it being difficult to obtain even as much as one drop of fluid in the pipette and what is obtained is composed partly of mucous. In some cases one may obtain two or three drops of a yellowish fluid which gives quite positive results in tests for bile, and occasionally the remains of copepods and other small animals may be found when microscopic examination of the contents is made. After 7 to 10 days of starvation, it is hard to get anything at all from the duodenum. Water is very seldom present in this part in fasting *Fundulus*.

The contents of the duodenum of fasting *Fundulus* is always alkaline to litmus, and its hydrogen ion concentration, as determined in more than 50 fishes ranges from pH 8.0 to pH 9.2. The variations in alkalinity depend chiefly on the presence or absence of bile, since bile taken directly from the gall-bladder has a hydrogen ion concentration of only pH 7.0 to 7.2. When mucus alone was present, the values were pH = 8.8 to pH = 9.2, whereas when bile also was present the values recorded were from pH = 8.0 to pH = 8.6. Regarding the possible presence and influence of sea water, we may say that samples taken from the sea-water pipe system at St. Andrews Biological Station were found in several determinations to have pH values of from 8.0 to 8.1. The con-

tents of the second and third parts of the intestine of freshly killed fasting fish were also always alkaline to litmus.

Experiments on Fed *Funduli*.

Although *Funduli* prefer animal food such as clam and the internal organs of dead fishes, even of their own species, they are practically omnivorous and will eat fish muscles, raw meat, bread,

Table I.

Digestion of Clam in *Fundulus*.

Date.	Fish No.	Contents of Intestine.	Reaction of the Contents.	
			pH.	Litmus.
July 17:	1	Yellowish fluid	8.8	Strong alkaline
9.30 A.M.	2	" "	8.6	" "
to	3	" mucus	8.8	" "
10.00 A.M.	4	Mucus and water	8.6	Alkaline
	5	Mucus	8.8	Strong alkaline
10.15 A.M.		12 pieces of clam were put in the aquarium; 6 pieces were eaten at once. At 12.00 noon two pieces were left, which were removed from the aquarium		
12.15 P.M.	3	Pieces of clam; no bile	8.4	Alkaline
	4	Scant; no bile	8.8	Strong alkaline
2.15 P.M.	1	Clam; bile	8.2	Alkaline
	2	" "	8.8	"
	3	Scant; "	8.6	"
	4	Nil.	8.6	"
	5	Clam; bile	8.4	"
7.15 P.M.	1	Nil.	8.4	"
	2	Clam; bile	8.2	"
	3	Mucus only	8.8	Strong alkaline
	4	" "	9.0	" "
	5	Clam; a little bile	8.8	" "
July 18:				
9.00 A.M.	5	Yellowish mucus	9.2	" "

hardboiled egg (yolk and white) and even watermelon. They digested perfectly well milk introduced by a pipette into the duodenum. The alimentary tract of freshly caught *Funduli* may contain mud, and small animals, insects, copepods, etc., but no matter what might be the nature of the food eaten or the stage of its

digestion, the reaction in the duodenum as well as in the second and third parts of the intestine was always alkaline, both in fish that were caught and investigated at once, on the spot, and also in those kept in aquaria and fed naturally or forcibly. However, the degree of alkalinity varied somewhat, according to the nature of the contents. As stated above, the reaction in the duodenum of fasting *Funduli* is decidedly alkaline, pH = 8.0 to 9.2. The presence of foods rich in protein, such as meat and clam, which are acid; of milk, which is neutral or only very slightly alkaline; of sea-water (pH = 8.2); and particularly the presence of considerable amounts of bile, which is also nearly neutral (pH = 7.0 to 7.2), all tend to reduce the degree of alkalinity.

Table I shows the course of digestion in *Fundulus* after natural feeding with clam. The contents of the duodenum were sucked out at certain intervals for each of five fishes. Prior to feeding they had been fasting for five days, and no excrements were found in the aquarium during the last two days of this period. Shortly before the fast was terminated, the pipette was introduced into the duodenum to withdraw any fluids which might be present.

Results analogous to those shown in the table were obtained with all the other kinds of food; the reaction in the duodenum was always alkaline.

The following experiment shows that there is an active discharge of bile into the intestine after a meal has been swallowed.

EXPERIMENT OF AUGUST 4.

In an aquarium in which nine fishes were kept for several days without food, at 8.30 A.M. there were placed several pieces of clams' flesh, but the amount was not sufficient to feed all of them. At 8.30 P.M. all of these fishes were killed and the contents of the duodenum and of the gall-bladder were investigated. In five fishes the duodenum was filled with clam and bile, while the gall-bladder was quite empty and collapsed. In the other four fishes the duodenum was empty, while the bladder was distended with bile.

In the case of injection of milk into the duodenum, the digestion was also always alkaline, the degree being somewhat less in the first hours (pH = 7.8) than in the later hours (pH = 8.8). Bile was present in the contents. The pH of the milk itself was pH = 7.2.

Alkaline reaction was also always found during the digestion of raw beef introduced into the duodenum. Six and one half hours after forcible feeding the contents were sucked out and the reddish-yellow fluid obtained gave an alkaline reaction, pH = 8.2.

Reaction to Various Stimuli.

Mechanical Stimulation.

Experiment of July 10.—As a means of mechanical stimulation, a few small pieces (2 mm. cubes) of cork or rubber were introduced into the duodenum in three *Funduli*, which were starved for at least three days. Just prior to the experiment the contents of the duodenum, consisting of a very little amount of mucus and some bile, were tested and found to be alkaline, pH = 8.6 to 9.0.

At 10.15 A.M., three pieces of cork were introduced into the duodenum of one fish; two pieces into that of the second; and two pieces of rubber into that of the third fish. The duodenal contents were sucked out at 2.15 P.M. and at 6.15 P.M. At both times, in each case, one could remove from 2 to 4 drops of water, with some mucus, but in only one fish was bile present, as indicated by the slightly yellowish colour of the contents. The reaction was in all cases alkaline, pH = 8.2 to 8.6. The next morning the corks were found in the aquarium, stuck together with mucus and probably had been ejected through the anal opening.

It is interesting to note that towards the end of the experiment water was found in the duodenum of these fishes, since its presence is quite unusual. From these experiments it appears that purely mechanical stimulation of the duodenum does not provide the necessary stimulus to provoke the pouring of bile into the intestine but that it does cause the fish to "drink water."

Hydrochloric Acid.

Table II, gives the results of introducing a few drops of 0.36 per cent. hydrochloric acid into the duodenum in two fishes that had been starved for 10 days. Its introduction did not provoke a flow of bile into the gut.

The table shows that in 6 hours after the introduction of 0.36 per cent. hydrochloric acid the duodenal contents had again become alkaline. It is interesting to note that in warm blooded animals the introduction of hydrochloric acid in the concentration in which

TABLE II.

INTRODUCTION OF HCl INTO DUODENUM.

Date and Time.	Fish No.	Contents of Intestine.	pH.	Litmus.
August 13:				
9.00 A.M.	1	Almost nil.	—	Alkaline
	2	" "	—	"
9.10 A.M.		Introduced 5 to 6 drops 0.36% HCl		
10.10 A.M.	1	Mucus and fluid	4.8	Acid
	2	" " "	5.0	"
11.10 A.M.	1	Mucus; very little fluid [1]	5.0	"
	2	" " " " [1]	5.4	"
12.10 P.M.	1	Mucus only [1]	5.0	"
	2	" " [1]	5.4	"
3.10 P.M.	1	Mucus and water	7.8	Very weak alkaline
	2	Watery fluid	7.6	" " "

it is in the gastric juice, *i.e.*, 0.5 to 0.36 per cent. practically does not provoke the entry of bile into the duodenum.

Alcohol.

From 4 to 6 drops of 5 per cent. pure ethyl-alcohol was introduced into the duodenum by means of a pipette 3 to 4 times per day. Four sets of analogous experiments were performed. In one of them, 2 fishes received alcohol regularly during 6 days. In the earlier experiments, in order to make sure that the alcohol reached the duodenum, it was mixed with a little finely ground carmin and in every case the red granules were afterwards found in the duodenal contents and also in the excrements.

The results of one of these experiments are shown in Table III. The fish had been starved for 2 or 3 days before the experiment, and the duodenum was almost empty. The very small amount of fluid present was alkaline to litmus. When the duodenum was evacuated 2 or more hours after injection of alcohol, it was found to contain only a very small amount of fluid, usually mucus. Except occasionally in the morning tests after a night

[1] To determine the reaction in these cases a few drops of distilled water were added to extracted material.

when the fish did not receive injections of alcohol, there was no bile present in the duodenum. The alkalinity of the contents was very high, pH = 8.8 to 9.2, but it was not determined whether this was due to the presence of pancreatic juice or of intestinal juice. The alcohol injected was slightly acid, pH = 6.8.

TABLE III.

INTRODUCTION OF ALCOHOL INTO THE DUODENUM.

Date and Time.	Contents of Intestine.	pH.	Litmus.	5% Alcohol Injected.
July 19:				
4.30 P.M.		—	—	4 drops
7.00 P.M.	1 drop of fluid	8.6	Alkaline	" "
July 20:				
9.30 A.M.	Mucus	9.0	"	" "
2.45 P.M.	2 drops	8.8	Strongly alkaline	" "
7.00 P.M.	2 drops	8.8	Alkaline	" "
July 21:				
11.25 A.M.	1 drop and mucus	8.2	Alkaline	" "
12.25 P.M.	3 drops	8.8	Strongly alkaline	" "
3.30 P.M.	2 drops	9.0	" "	" "
7.15 P.M.	2 drops	9.0	" "	None
July 22:				
11.50 A.M.	Mucus only [1]	9.4	" "	4 drops
July 23:				
9.45 A.M.	Nil.	—	—	" "
11.50 A.M.	3 drops	8.8	Strongly alkaline	" "
2.45 P.M.	1 drop and mucus	9.0	" "	" "
July 24:				
9.15 A.M.	Mucus only [1]	9.2	" "	" "
12.15 P.M.	1 to 2 drops	9.2	" "	None

N. B. Bile was not found in the contents during this experiment.

It is important to note that the experiments with alcohol afford further evidence that gastric glands are absent in Fundulus. Although alcohol is one of the strongest stimuli for the secretion of gastric juices, in our experiments the intestinal contents never had an acid reaction, but, as stated above, were always highly alkaline.

Pilocarpin.

Table IV. gives the results of one of eight sets of experiments with pilocarpin. Five or 6 drops of 1 per cent. solution of pilocarpin was injected into the duodenum of two fishes which had

[1] To determine the reaction in these cases a few drops of distilled water was added.

been starved for 7 days. At the end of this period the duodenum contained in each case a small amount of mucus and fluid which was strongly alkaline, pH = 9.2 and 9.0 respectively.

The interesting feature of the experiments is that pilocarpin provoked chiefly the motor phenomena of the alimentary canal. Its introduction into the duodenum excited evacuation of the bowel and this was accompanied by the entry of the bile into the intestine. The contents of the duodenum, extracted one hour after injection of the pilocarpin, had a yellowish or greenish colour and gave a positive test for bile, with flowers of sulphur. Following the injection, the alkalinity of contents of the duodenum was comparatively low, pH between 7.8 and 8.6.

TABLE IV.

INTRODUCTION OF PILOCARPIN INTO THE DUODENUM.

Date and Time.	Contents of Intestine.	pH.	Litmus.
August 10:			
8.35 A.M.	1. Mucus and some fluid	9.2	Alkaline
	2. " " " "	9.0	"
8.45 A.M.	Injected 5 to 6 drops of 1% sol. of pilcarpin into each fish		
9.45 A.M.	1. 2 to 3 drops of emerald green bile	8.0	Weakly alkaline
	2. 8 drops of green fluid; re-injected 6 drops of it	7.8	" "
10.45 A.M.	1. 2 to 3 drops of light green fluid	8.4	" "
	2. 10 drops of green fluid; re-injected 8 drops of it	8.2	" "
11.45 A.M.	1. 2 to 3 drops of light green fluid	8.4	Alkaline
	2. 8 drops of green fluid	8.2	Weakly alkaline
3.15 P.M.	1. 4 to 5 drops of a very light green fluid with mucus	8.6	Alkaline
	2. 3 to 4 drops of clear, colorless fluid	8.6	"

Atropin.

The injection of 1 per cent. atropin sulphate into the duodenum, as one would expect, inhibited the intestinal secretions (it was hard to get anything but mucus from the intestine) and also prevented the entry of bile into the gut. Table V. gives the results of experiments on two fishes which had been starved for 10 days.

The reaction of the duodenal contents was usually strongly alkaline.

It is interesting to note that one hour after the introduction of atropin into the intestine, the skin of the fish became dark, almost black. In five hours the normal colour of the skin had returned. These changes were not due to light effects, as the whole day was grey and cloudy.

From the foregoing experiments it may be seen that the first part of the alimentary canal of Fundulus may properly be designated the duodenum, since it corresponds to the duodenum of vertebrates possessing a stomach.

TABLE V.

INTRODUCTION OF ATROPIN INTO THE DUODENUM.

Date and Time.	Contents of Intestine.	pH.	Litmus.
August 13:			
9.10 A.M.	1. Nil.	—	Alkaline
	2. "	—	"
9.20 A.M.	Injected 4 to 5 drops of 1% sol. of atropin sulphate into each fish		
10.20 A.M.	1. 2 to 3 drops of fluid, with mucus	9.0	Strongly alkaline
	2. 1 drop of fluid, with mucus	8.8	Alkaline
11.20 A.M.	1. Very little fluid and mucus; difficult to obtain it	8.8	"
	2. Very little fluid and mucus; difficult to obtain it	8.8	"
12.20 P.M.	1. Almost nil.; a little mucus	8.8	Strongly alkaline
	2. Almost nil.; a little mucus	8.8	Alkaline
3.20 P.M.	1. Mucus and 1 to 2 drops of water	9.0	Strongly alkaline
	2. Mucus and 3 drops of water	8.8	" "

N. B. There was no bile present in the intestinal contents during the whole experiment.

ENZYMES OF THE MUCOUS MEMBRANE OF THE FIRST PART OF THE INTESTINE.

The only method which we applied to the study of the enzymes of this mucous membrane was the reaction of its extract on various media. The imperfections of this method are very well known. A certain possibility that we might have to consider autolytic enzymes, as well as digestive enzymes, was suggested by the ob-

servations of Bradley (1922[6]), on autolysis of liver and kidney. He produced evidence to show that in autolysis there are two autolytic enzyme-complexes, one of which is analogous to trypsin, but has its optimum activity at pH 4 to 4.5, whereas true trypsin has its optimum activity at pH 8. Beyond the range from pH 7 to pH 3, the action of this autolytic enzyme-complex is insignificant, while for trypsin the range of activity is pH 9 to pH 4.5. The other autolytic enzyme-complex is analogous to pepsin, but has its optimum reaction at pH 4.5, whereas pepsin has its optimum at pH 1.5. It is destroyed at pH 2.6, while pepsin is very active at that degree of acidity. The range of activity for pepsin is from pH 0.5 to pH 6.5 (McFarlane, et al.[7]).

By adjusting the reaction of the media to hydrogen ion concentrations which are beyond the limits of activity for any autolytic enzyme-complexes which might be present, one could expect to obtain reactions due solely to digestive enzymes. To minimize the possibilities of contamination all extracts of the intestinal mucous membrane were kept under toluene and all test tubes and pipettes were sterilized before use.

The extracts from the mucous membrane usually of the duodenum were prepared in the following way. The intestine, cut out from the body, was cleaned from all surrounding tissues, split longitudinally, and washed several times in tap water. Only the mucous membrane was scraped by a knife and mixed with the corresponding fluid. The temperature in the incubator was 35° C.

For use in the various experiments extracts of the mucous membrane of the intestine were made with 30 per cent. alcohol, 0.9 per cent. NaCl, glycerine, and 0.36 per cent. HCl. These were used at various intervals after their preparation and were kept under various conditions of temperature.

In preparing the extracts for use they were filtered, or otherwise separated from the tissue cells, and were then diluted with 3 or 4 volumes of distilled water. The hydrogen ion concentration of the mixture was adjusted to the desired level by adding either 0.36 per cent. HCl or dry sodium carbonate. All tests of enzymatic action reported here must be looked upon as qualitative only.

Action of the Extracts on Protein (Fibrin).

For the experiments on the digestion of protein, fibrin was extracted from fresh blood and was then kept in glycerol. Before use it was thoroughly washed; dried between leaves of blotting paper; and then minced and small portions of it were placed in several test tubes along with the extract to be tested and a few drops of toluene. The whole mixture was incubated at 35° C.

In order to rule out the problem of autolysis the reaction of the media during these tests was adjusted to H ion concentrations of pH = 2; pH = 8.0; pH = 8.4 and pH = 9.0 respectively.

No trace of digestion of fibrin was manifested in periods ranging from 48 to 62 hours respectively, a fact which indicates that the extract of intestinal mucous membrane contains neither pepsin nor trypsin.

In one case, on the sixth day the fibrin was found to be partly dissolved. The initial pH of the mixture in this case was 8.4, but it may have been that during such a long period of incubation the medium may have lost its alkalinity to such an extent that the autolytic enzyme-complexes had started to act.

Action of the Extracts on Peptone.

In one set of experiments a very gradual but not complete discoloration of a mixture of purified casein, phenol red, and saline extract was observed during 24 hours in the incubator. The initial pH of the mixture was 8.0.

In another set of experiments a 2 per cent. solution of Witte's peptone was mixed with boiled, and with unboiled, saline intestinal extract, respectively, and was adjusted to pH = 7.8. After 3 days in the incubator biuret tests showed that there was less peptone in the mixture with fresh extract, than in the control, in which boiled extract was used, indicating that the fresh extract had a weak ereptic action.

Action of the Extracts on Starch.

Table VI. gives the results of two experiments in which the extract of the intestinal mucous membrane on normal saline was allowed to act on starch. In one case fresh extract was used and in the other the extract was boiled. In each case the mixture was

adjusted to pH 6.7 and 2 drops of toluene were added, after which it was placed in the incubator. In the subsequent test for starch a weak solution of iodine in potassium iodide was employed. The table shows that, whereas the tests with boiled extract were negative, those with fresh extract gave a progressive diminution in the amount of starch present and a corresponding increase in the sugar content of the mixture, indicating a decided amylolytic action.

That the amylolytic action of the intestinal extract was due to the enzyme contained in the mucous membrane and not to the pancreatic juice, which could be fixed on the intestinal mucous membrane, in spite of its thorough washing, is seen from the fact that proteolytic action was absent in the same extracts.

TABLE VI.

AMYLOLYTIC ACTION OF EXTRACT OF THE INTESTINAL MUCOUS MEMBRANE.

Date.	Experiment 1.	Experiment 2.
August 4.....	3 cc. of 2% soluble starch 10 drops boiled extract pH = 6.7 Iodine reaction—blue	3 cc. of 2% soluble starch 10 drops fresh extract pH = 6.7 Iodine reaction—blue
3.30 P.M.....	Placed in incubator	Placed in incubator
August 5:		
8.30 A.M.....	Iodine—no change Fehling—negative pH = 6.7	Iodine—light purple Fehling—positive pH = 6.7
7.30 P.M.....	Iodine—no change	Iodine—light purple
August 6:		
10.15 A.M.....	Iodine—no change Fehling—no change pH = 6.6	Iodine—very light purple Fehling—strongly positive pH = 6.7
7.30 P.M.....	—no change	—progressive change
August 7:		
7.30 P M.....	Iodine—no change Fehling—no change pH = 6.6	Iodine—almost colorless Fehling—strongly positive pH = 6.7

Action of the Extracts on Lipoids.

It was found that unboiled extract of the intestinal mucous membrane had a weak lypolytic action on olive oil and a somewhat more pronounced effect on cream. The reaction on cream is shown in Table X., experiments 5 and 6.

Enterokinase of the Extract.

In addition to its action as a ferment acting directly on peptones, starch and fats, it was shown that the unboiled intestinal extract had also the power of greatly accelerating the tryptic action of liver extract of *Fundulus* and of the bile: to a lesser degree, this extract increased the action of lipase on the digestion of fat, whereas boiled extracts had no such effects.

Activation of Trypsinogen.

Table VII. shows the results of adding unboiled saline intestinal extract to a mixture containing minced fibrin and glycerin extract

TABLE VII.

ACTIVATION OF TRYPSINOGEN BY EXTRACT OF INTESTINAL MUCOUS MEMBRANE.

Date.	Exp. 1.	Exp. 2.	Exp. 3.
August 13:			
12.00 Noon	Minced fibrin 8 dr. glycerin extract of hepatic pancreas 12 dr. water No intestinal extract (control) pH = 8.2	Minced fibrin 8 dr. glycerin extract of hepatic pancreas 10 dr. water 2 dr. intestinal extract (in saline) pH = 8.2	Minced fibrin 8 dr. glycerin extract of hepatic pancreas; and intestinal mucous membrane prepared a day before 12 dr. water 2 dr. intestinal extract (in saline) pH = 8.2
1.15 P.M.	No change	No change	Completely digested
3.30 P.M.	" "	Digestion begun	—
5.30 P.M.	" "	Half digested	—
6.30 P.M.	" "	Completely digested	—
August 14:			
9.00 A.M.	" "	—	—
August 15:			
9.00 A.M.	" "	—	—
August 16:			
8.30 A.M.	Marked digestion	—	—
1.00 P.M.	Half digested	—	—
3.00 P.M.	Completely digested	—	—
			N.B. In this experiment (3) the extracts of hepatic pancreas and of intestine were mixed together on the previous day

of the liver of *Fundulus*. (As stated above, the liver of these fishes is invaded by pancreatic tissue, so that extracts might contain considerable trypsinogen.) In Experiment No. 3, the fibrin was very quickly digested. The probable cause was that the liver extract and the extract of the intestine had been mixed together on the previous day and that in the interval the trypsinogen had already become active trypsin. In Experiment No. 2, the activation of protrypsin required a latent period of about 3 hours. The presence of glycerin in the mixture probably inhibited the process of activation. In Experiment No. 1, intestinal extract was not used, and, therefore, digestion did not take place until the third day. The most probable explanation of this phenomenon is that it required such an interval for the spontaneous activation of the protrypsin.

Other experiments which demonstrate the activation of trypsinogen by intestinal extracts are recorded in Table IX.

Acceleration of Lipolysis.

The experiments in which the digestion of fat was accelerated by the addition of unboiled intestinal extract to a mixture containing bile and cream are recorded in Table X. and in Table XI.

Enzymes in Gall-bladder Bile.

At the time of these experiments, the histological investigations had not been made and, therefore, it was not then known that the gall-bladder of *Fundulus* is surrounded by a thin layer of pancreatic tissue, the presence of which introduces the possibility that a small amount of pancreatic enzyme may have become mixed with the bile during the manipulations for emptying the gall-bladder.

The gall-bladder was dissected out and removed from the body with sterile instruments, the bile was pressed out of the viscus, and the contents were collected in a sterile container. The bile secured in this manner contained a very active amylase, trypsinogen and prolipase. The last two substances could be activated by the unboiled extract of intestinal mucous membrane.

Table VIII. gives the results of two experiments to demonstrate the amylolytic action of fresh bile.

TABLE VIII.

AMYLOLYTIC ACTION OF GALL-BLADDER BILE.

August 7:

Time.	Experiment 1.	Experiment 2.
9.30 A.M.....	2 cc. of 2% soluble starch 2 drops boiled bile pH = 6.6	2 cc. of 2% soluble starch 2 drops fresh bile pH = 6.6
9.40 A.M.....	Placed in water bath at 35° C.	Placed in water bath at 35° C.
10.40 A.M.....	Iodine—dark blue Fehling—negative	Iodine—purple color Fehling—positive
11.40 A.M.....	No change	Iodine—light purple Fehling—strongly positive
2.40 P.M.....	No change pH = 6.6	Iodine—very light purple almost colorless Fehling—strongly positive pH = 6.6

TABLE IX.

TRYPSINOGEN OF THE BILE AND ITS ACTIVATION BY THE EXTRACT OF THE INTESTINAL MUCOUS MEMBRANE.

Date and Time.	Experiment 1.	Experiment 2.	Experiment 3.
August 5:	Minced fibrin 2 cc. bile 8 cc. dist. water No extract (control) pH = 8.3	Minced fibrin 2 cc. bile 8 cc. dist. water 1 drop boiled intestinal extract pH = 8.3	Minced fibrin 2 cc. bile 8 cc. dist. water 1 drop fresh intestinal extract pH = 8.2
10.20 A.M.	Put in incubator	Put in incubator	Put in incubator
12.05 P.M.	No change	No change	Digestion begun
1.15 P.M.	" "	" "	Half digested
2.00 P.M.	" "	" "	Completely digested
7.30 P.M.	" "	" "	—
August 6:			
10.20 A.M.	" "	" "	—
	Added 1 drop fresh intestinal extract	Added 1 drop fresh intestinal extract	
11.30 A.M.	Digestion begun	Digestion begun	
12.05 P.M.	Half digested	Half digested	
1.00 P.M.	Completely digested	Completely digested	

Table IX. shows that the bile alone or in the presence of boiled intestinal extract has no digestive action on fibrin, but when fresh

TABLE X.

PROLIPASE IN GALL-BLADDER BILE LIPASE AND ENTEROKINASE IN EXTRACTS OF INTESTINAL MUCOUS MEMBRANE.

Date.	Experiment 1.	Experiment 2.	Experiment 3.	Experiment 4.	Experiment 5.
Aug. 12.....	Cream substrate (control)	Cream substrate 4 dr. bile	Cream substrate 4 dr. bile 2 dr. fresh intestinal extract	Cream substrate No bile 4 dr. boiled intestinal extract	Cream substrate No bile 4 dr. fresh intestinal extract
3.00 P.M.....	Put in incubator	Put in incubator	Put in incubator	Put in incubator	Put in incubator
4.30 P.M.....	No change	Light purple 1 dr. NaOH added	Light green 2 dr. NaOH added	No change	No change
6.00 P.M.....	" "	Purple	Light green 3 dr. NaOH added	" "	" "
8.00 P.M.....	" "	Light green 1 dr. NaOH added	Light purple 1 dr. NaOH added	" "	Light pink
Aug. 13: 8.00 A.M.....	" "	Light green 3 dr. NaOH added	Grey 2 dr. NaOH added	" "	White 1 dr. NaOH added
3.00 P.M.....	" "	Grey-purple 1 dr. NaOH added	Pink-purple 1 dr. NaOH added	" "	Pink
NaOH solution added during 24 hours	Nil.	6 drops	9 drops	Nil.	1 drop

TABLE XI.

PROLIPASE IN THE GALL-BLADDER BILE, ITS ACTIVATION BY THE INTESTINAL EXTRACT.

Date.	Experiment 1.	Experiment 2.	Experiment 3.	Experiment 4.	Experiment 5.
Aug. 16.....	Cream substrate (control)	Cream substrate 5 dr. of boiled bile	Cream substrate 3 dr. of fresh bile	Cream substrate 2 dr. boiled bile and 2 dr. boiled intestinal extract	Cream substrate 2 dr. of fresh bile and 2 dr. of fresh intestinal extract
11.00 A.M.....	Put in incubator	Put in incubator	Put in incubator	Put in incubator	Put in incubator
12.00 Noon.....	No change	No change	Light pink 1 dr. NaOH added	No change	Light pink 1 dr. NaOH added
1.00 P.M.....	" "	" "	No change	" "	Light pink 1 dr. NaOH added
2.00 P.M.....	" "	" "	Very little change	" "	Grey 1 dr. NaOH added
5.00 P.M.....	" "	" "	No change	" "	Grey 3 dr. NaOH added
8.30 P.M.....	" "	" "	" "	" "	Grey 3 dr. NaOH added
Aug. 17: 8.00 A.M.....	" "	" "	Grey purple 1 dr. NaOH added	" "	Light pink 2 dr. NaOH added
NaOH solution added during 17 hours	Nil.	Nil.	2 drops	Nil.	11 drops

extract is used, tryptic digestion takes place in a short time. Apparently trypsinogen was present in the bile, and was activated by the fresh intestinal extract.

To these findings might also be added the fact that when toluene is not used, self activation of the tryptic enzyme in the bile takes place after one or two days at room temperature, and much more quickly in the incubator.

Tables X. and XI. show that the gall-bladder bile contains a prolipase, which may be activated by the extract of the intestinal mucous membrane. From the same tables we learn too that the intestinal extract possesses a weak lipolytic action and contains an activator for the bile lipase.

In all of the experiments recorded in Tables X. and XI. boiled cream, diluted with one volume of distilled water was used as the substrate, and to this was added one drop of 1 per cent. phenolphthalein and sufficient 0.09 per cent NaOH solution (2 to 4 drops) to produce in each case a distinct pink color ($pH = 8.3$). This alkaline pink mixture of diluted boiled cream is referred to in the table as the cream substrate. After decoloration of the mixture in the incubator, a sufficient amount of 0.09 per cent. NaOH solution was added to restore its initial pink color.

From Tables X. and XI. one may see that: the bile alone possesses a weak lypolitic action which develops slowly (Exp. 2, Table X. and Exp. 3, Table XI.); this action is due to an enzyme, because boiled bile loses its lypolitic action (Exp. 2, Table XI.); the intestinal extract alone has a weak lipolytic action (Exp. 5, Table X.); boiled intestinal extract loses its enzymatic action (Exp. 4, Table X.); when bile and intestinal extract are combined the lipolysis is much accelerated (Exp. 3, Table X. and Exp. 5, Table XI.); boiling destroys the lipolytic action and the activation (Exp. 4, Table XI.).

It is evident that the result of combined action of the bile and of the intestinal extract is not merely a simple summation of their activities, but indicates rather that the intestinal extract possesses a substance which activates the bile viz. pancreatic lipase. There is also a possibility that the bile increased the action of the intestinal lipase.

Since the presence of pancreatic tissue on the gall-bladder makes

it possible that enzymes of the pancreas may have become mixed with the bile used in these experiments, the latter must necessarily be repeated with bile obtained by more adequate methods. If it can be demonstrated that the enzymes in question were admixed to the bile with the pancreatic cells surrounding the gall-bladder in *Fundulus*, these experiments will offer a proof that the pancreas of this animal elaborates the same enzymes as the pancreas of warm-blooded animals, two of them in form of zymogen (protrypsin and prolipase), and one in active form (amylase).

Discussion.

The data reported in this investigation show that *Fundulus heteroclitus* lacks a stomach, the intestine joining directly to the œsophagus. Since there are several species of fishes with an analogous structure of the alimentary canal, from the point of view of their digestive system, all fishes may be divided into two main groups, namely, (1) those possessing a pepsin-hydrochloric acid digestion, and (2) those lacking it. This makes it possible to differentiate two types of digestion in fishes; namely, acid-alkaline, and exclusively alkaline. There is very little doubt that these decidedly different types of digestion greatly affect the internal chemisms of the body, in the two cases; because, in the one case, important changes in the chemical composition of the body fluids are provoked following the secretion of acid into the stomach. Similar changes must necessarily be quite absent in fishes deprived of a stomach and must be greatly attenuated in the pathological syndrome, known as achylia gastrica, or in animals with experimentally removed stomachs. Therefore, the study of the animals of a type of *Fundulus* presents an interesting problem.

Summary.

1. That *Fundulus heteroclitus* does not possess a stomach is shown by the following evidence:

(*a*) There is absolute absence of pepsin and hydrochloric acid in the digestive juices.

(*b*) Every phase of digestion takes place in an alkaline medium.

(*c*) The first part of the intestine (duodenum) is joined directly to the œsophagus.

(*d*) The bile and pancreatic juice are poured into the gut only a few millimeters (3 or 4) below the œsophagus.

(*e*) The duodenum is capable of dilating to form a container for food.

2. Extracts of the intestinal mucous membrane manifest an amylolytic, a lipolytic and an ereptic digestive action. They probably contain also an enterokinase.

3. The gall-bladder bile contains a trypsinogen, a prolipase and an amylase. The origin of these enzymes must be investigated further, because they could be admixed to the bile from the layer of pancreatic tissue surrounding the gall-bladder, during its collection.

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